

Secondary metabolites from the Bermudian sponge Tedania ignis
by Rhonda Lynn Dillman

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Chemistry
Montana State University
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Abstract:
Although extensive research has been done with many of the sponge genera, relatively little has been reported for the genus Tedania. To further investigate this genus, Tedania ignis was collected off Bermuda and extracted. Herein is reported the isolation of seventeen metabolites, eight of which are novel and most of which had not been previously isolated from a marine source.

In all, three diketopiperazines, four indoles, a carbazole, a β-carboline, four benzenoid compounds, a polybrominated diphenyl ether and three polybrominated dibenzo-p-dioxins were isolated. The polybrominated compounds represent the first halogenated molecules reported from Tedania. The biological activity of these compounds was evaluated in antimicrobial, brine shrimp (cytotoxicity), phytotoxicity and plant growth assays and these results, along with the biological significance of the compounds, are discussed.

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A thesis submitted in partial fulfillment
of the requirements for the degree

of

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in

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APPROVAL

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Rhonda Lynn Dillman

This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

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Date 7/16/90

This thesis is dedicated to my parents, Marie and Richard Dillman, for all their love, support and encouragement.

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ABSTRACT

Although extensive research has been done with many of the sponge genera, relatively little has been reported for the genus ***Tedania***. To further investigate this genus, ***Tedania ignis*** was collected off Bermuda and extracted. Herein is reported the isolation of seventeen metabolites, eight of which are novel and most of which had not been previously isolated from a marine source. In all, three diketopiperazines, four indoles, a carbazole, a β-carboline, four benzenoid compounds, a polybrominated diphenyl ether and three polybrominated dibenzo-p-dioxins were isolated. The polybrominated compounds represent the first halogenated molecules reported from ***Tedania***. The biological activity of these compounds was evaluated in antimicrobial, brine shrimp (cytotoxicity), phytotoxicity and plant growth assays and these results, along with the biological significance of the compounds, are discussed.

INTRODUCTION

While the study of the secondary metabolites of terrestrial plants began in the 1800's, it was not until after World War II, when man developed the technology to explore the ocean, that the study of marine life blossomed. The 1960's saw the beginning of what is today modern marine natural product chemistry (1).

Natural product chemists endeavor to explore the metabolites of a variety of organisms. All organisms produce both primary and secondary metabolites, the difference between which is fairly straightforward. Primary metabolites are often defined as the essential metabolites of an organism: the amino acids, sugars, nucleotides, etc. They are ubiquitous, being found unchanged throughout most genera and species (2).

Secondary metabolites are usually end products of the primary metabolic processes and more narrowly distributed. Hence, they can be characteristic of certain genera and species. Secondary metabolites are considered to be non-essential in the sense of maintaining primary life functions. They can, however, be extremely important in

helping an organism to survive, for example, predation or disease (2).

The diversity of secondary metabolites is enormous, as is the range of their biological activities. The terpenoids, acetogenins, aromatics and alkaloids represent a wide variety of molecules with wide-ranging biological activities (1). While natural product chemists have discovered a variety of biological activities for isolated compounds that are beneficial to man, the actual purpose of many of these secondary metabolites within the organism in its natural environment can only be hypothesized.

Due to the wide distribution of the same primary metabolites throughout both terrestrial and marine organisms, similar and often identical secondary metabolites are found to occur. As one would expect, differences between the two environments can lead to the production of distinct types of compounds. One of the largest variables between the two environs is the extreme salinity of the marine world.

While halogenated compounds are relatively rare in the terrestrial environment, notable exceptions do exist (3). Terrestrial microorganisms seem to be the biggest producers of chlorinated metabolites, such as the antibiotics griseofulvin and chlorotetracycline (4).

Brominated compounds are rarely seen in terrestrial organisms, although some microorganisms will produce brominated analogues of their chlorinated metabolites (e.g., griseofulvin) when bromine is added to their culture media (4). It is not unreasonable, then, that these bromine analogues may occur naturally, only in too small a quantity to be isolated. In fact, a ***Xanthomonas*** species has been shown to produce mono- and dibrominated pigments in media not artifically enriched with bromine (5).

The story is quite different in marine organisms. In an environment where the concentrations of chlorine and bromine are 19,000 mg/l and 65 mg/l, respectively, the metabolism of halogenated compounds may be a means of adapting to the salinity (3). But while one would expect a higher proportion of chlorinated compounds due to its higher concentration, brominated compounds seem to predominate. This is due to the ability of algae to accumulate bromine and the lower oxidation potential of bromine versus chlorine (3).

Our investigation of the Bermudian sponge, ***Tedania ignis***, has revealed some interesting and novel secondary metabolites. It was the goal of this research to characterize these compounds and to determine their

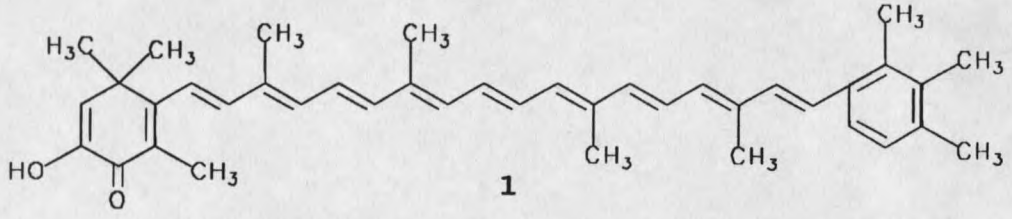
biological activity through several in house assays. The question of whether or not all these metabolites are attributable to the sponge itself will be addressed.

BACKGROUND ON THE SPONGE *Tedanis ignis*

The phylum Porifera contains four classes and over 5000 species of sponges, which vary greatly in appearance and habitat. Sponges are the most primitive multicellular organisms known. They are sessile and hence dependent on water currents to supply nutrients and to remove waste (6).

Hundreds of metabolites have been isolated from marine sponges. When compared to other sponge genera, relatively few compounds have been reported from the genus ***Tedania***. Only three species are noted in the chemical literature, these being ***T. ignis***, ***T. digitata*** and a ***Tedania*** sp.

The investigation of ***T. digitata*** has yielded only a series of carotenoids including tedanin, **1** (7,8), tedaniaxanthin, **2** (9), isotedaniaxanthin, **3** (10), and 7,8-didehydroaaptopurpurin, **4** (11).



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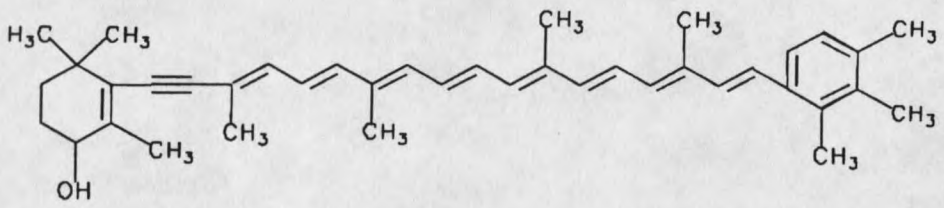

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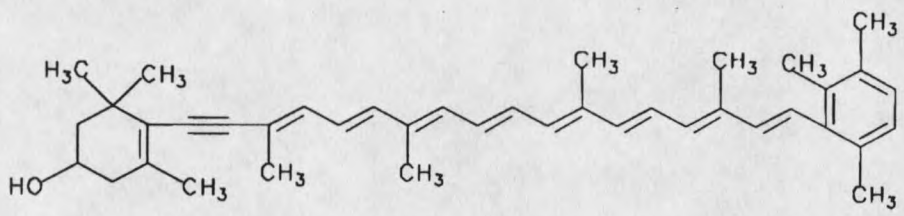

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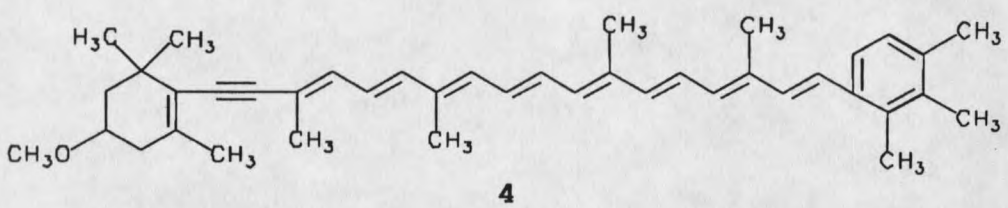

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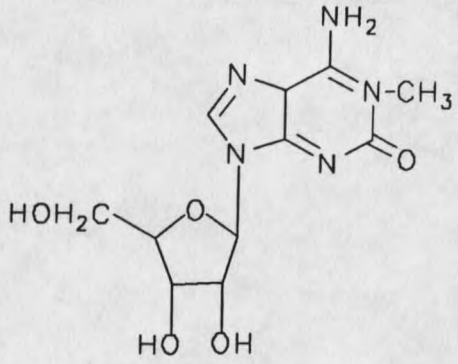
Cook and co-workers isolated the nucleoside, 1-methylisoguanosine, 5, from an Australian ***Tedania*** sp. (12). It reportedly has numerous biological activities, including muscle relaxant, central nervous system, antiinflammatory, antiallergenic and hypotensive activities (13).



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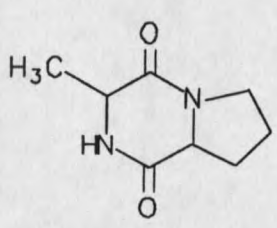
By far, ***T. ignis*** is the most interesting of the three species. It is a bright orange sponge found in the shallow waters of Bermuda and the Caribbean and is commonly referred to as the fire sponge because of the dermatitis that occurs upon contact (14).

Schmitz and co-workers began an investigation of ***T. ignis*** in response to the cytotoxicity and tumor inhibition activity they found in several of the sponge extracts. The first of the compounds to be isolated, but not those responsible for the activity seen in the extracts, were the diketopiperazines **6**, **7**, and **8** (15). These compounds had been isolated previously from plants and fungi (16, 17, 18). Schmitz reported the yield of the diketopiperazines to be very small and suggested that they were actually endosymbiotic microbial metabolites. This theory was strongly supported when the same

diketopiperazines were isolated from a ***Micrococcus*** sp. cultured from ***T. ignis*** (19).



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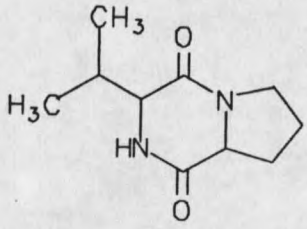

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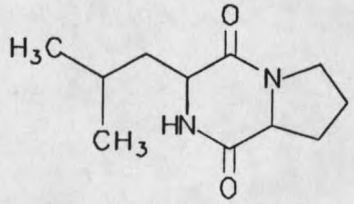

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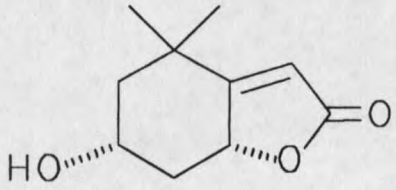
Two mildly cytotoxic compounds (KB: ED_{50}= 21 μg/ml) were found in the organic extract (15). These were epiloliolide, **9**, and atisane-3β,16α-diol, **10**. Neither compound had previously been isolated as a natural product.



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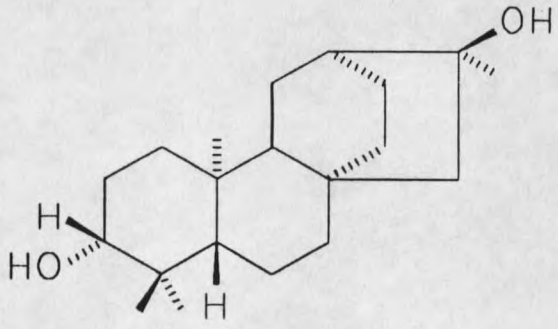

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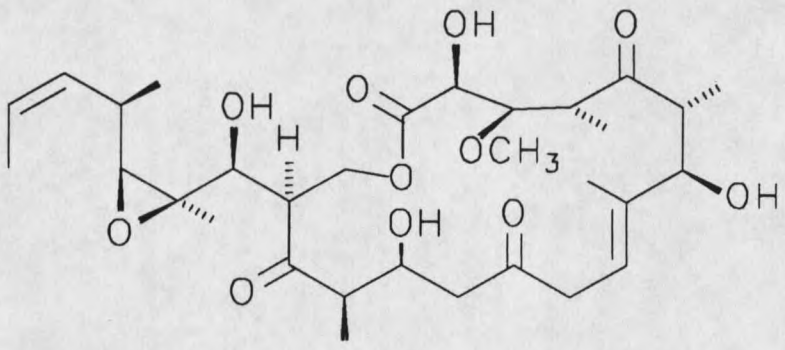
The most interesting compound isolated from ***T. ignis*** was the macrolide tedanolide, **11** (20). The compound was

found to be extremely cytotoxic with an ED_{50} in KB=2.5 x 10^{-4} µg/ml. "Active" fractions are those with an $ED_{50} \leq 20$ µg/ml (21). Tedanolide was isolated in very low yield (1 x 10^{-4}% of dry weight) and Schmitz once again suggested a microbial origin (20).



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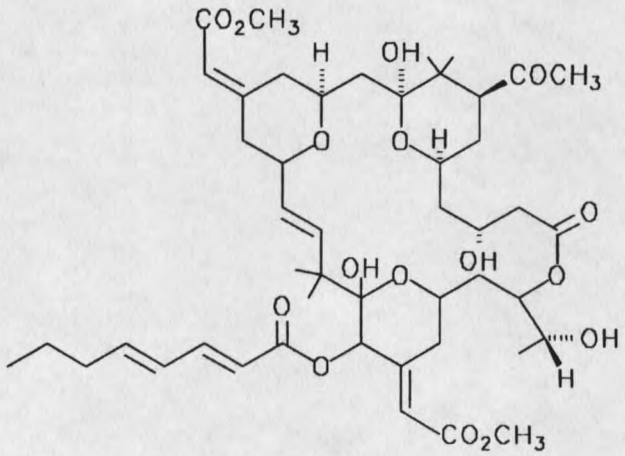
Sponges are known to harbor symbiotic organisms (6) and, within the past five years, several sponge metabolites have been shown to be the products of endosymbiotic microorganisms. One such metabolite is okadaic acid, which had been isolated from the sponge ***Halichondria okadai*** (22), but which was actually produced by a dinoflagellate (23). Another example is from ***T. ignis*** itself. Two compounds identified only as fα and fβ were isolated by Sevcik and Barboza and found to have presynaptic effects on frog muscle (24). These compounds

were later discovered to be toxins produced by a phytoplankton living within ***T. ignis*** (25,26). Other sponge metabolites suspected of being microbial in origin include the hexachlorinated metabolites dysidenin and isodysidenin from the sponge ***Dysidea herbacea*** (27) and sterols isolated from ***D. herbacea*** (28).

Statement of Objectives and Goals

In the summers of 1986 and 1987, our research group collected numerous marine invertebrates off the coast of Bermuda. We targeted organisms whose known metabolites were suspected of being produced by endosymbiotic microorganisms. These included the bryozoan ***Bugula neritina***, from which the bryostatins, e.g. **12**, were isolated (29) and the sponge ***Tedania ignis***, from which tedanolide, **11**, was isolated (20).



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Each of the invertebrates were cultured for endosymbiotic microorganisms. This was accomplished by excising small pieces of internal tissue, which were then placed on marine agar in Petri dishes. As microbial growth appeared, colonies were transferred until pure cultures were established. Using this technique, 13 isolates were obtained from ***Bugula*** and 33 isolates were obtained from ***Tedania***.

It was our goal to find a microorganism that produced one of these known metabolites. We reasoned that the easiest way to find the isolate responsible for producing the compound in question was to develop a simple means of detecting the compound in a crude extract.

Work began on ***T. ignis*** in hopes of isolating tedanolide. A TLC spray that would detect the compound in a crude extract would then be developed. The crude extract from a culture of each microbial isolate from ***T. ignis*** would then be tested with the spray.

Unfortunately, tedanolide was never found. Instead, several other metabolites, not previously reported from ***T. ignis***, or, in some cases, any other marine source were isolated.

The fact that one of the isolated metabolites had also been reported from a ***Tedania*** microbe was strong

evidence supporting a microbial origin for several of these metabolites. It became our goal to find other compounds that had also been isolated from the microorganism. Although these were not found, several metabolites of possible microbial origin were isolated.

Since relatively little work had been done on ***Tedania***, it was believed that some new and interesting compounds could be found in the sponge. As always, the focus of attention was on fractions exhibiting biological activity. In this case, these fractions seemed to contain mostly aromatic compounds. The objective was to purify and identify these metabolites, determine their biological activity and to make some educated hypotheses about their origin.

Extraction and Isolation of *Tedania* Metabolites

T. ignis was collected in the shallow coastal waters off Bermuda in the fall of 1979 and stored in acetone. After storage at 5°C, the acetone-water was removed by filtration. The sponge was ground in MeOH and then soaked in CH_2Cl_2. The organic extracts were combined and further partitioned among hexane, CCl_4, $CHCl_3$, ethyl acetate and water.

Isolation and purification of the metabolites

proceeded using brine shrimp toxicity guided fractionation. The CCl_4 and $CHCl_3$ crudes from the solvent partition were chromatographed, separately, on BioBeads S-X4. Several active fractions from each were then applied, separately, to Sephadex LH-20. Final purification of the metabolites involved separations using centrifugal countercurrent chromatography, low pressure silica gel, Sephadex LH-20 and BioBeads S-X8 chromatographies.

Countercurrent chromatography (CCC) proved to be invaluable during this research. It is a relatively new technique that has certain advantages over adsorption chromatography. CCC is based on liquid-liquid partitioning using two-phase solvent systems and hence, involves no solid support to which compounds may adsorb. This proved to be a distinct advantage in this investigation where many fractions contained only a few milligrams and any loss of material would have been detrimental. Another advantage to CCC was the shorter time period needed per separation. Where a CCC separation would average 1-2 hours, adsorption chromatographies averaged ~6 hours.

CCC was especially useful in the separation of similar compounds. During this research, the technique was used in the separation of diketopiperazines, indoles,

and isomers of a polybrominated hydroxydibenzo-p-dioxin. Separation of these isomers was first attempted using several adsorption chromatography systems, all of which failed. However, as will be seen later, CCC gave good resolution of the isomers in a short period of time.

Structure Elucidation of Diketopiperazines

With the knowledge that diketopiperazines had previously been isolated from both ***T. ignis*** (15) and a bacterium endosymbiotic to ***T. ignis*** (19), only a cursory look was at first given to the fractions containing these compounds. Cyclo(Pro-Val), 7, had been isolated from both of the above sources and was easily identified by ^{1}H NMR and MS data. Several of the NMR peaks (Fig. 1) were common to the proline moiety of the diketopiperazines. These included the ring juncture methine at δ4.08 (1H, t) and the three methylene groups at δ3.70-3.50 (2H, m), δ2.35 (2H, m) and δ2.10-1.80 (2H, m). The rest of the signals in the spectrum matched those for valine. These included a ring juncture methine at δ3.92 (1H, d), a methine at δ2.62 (1H, m) and two methyl doublets at δ1.08 and δ0.90. MS analysis confirmed the structure with a molecular ion at 196 (Fig.2). Cleavage of the valine

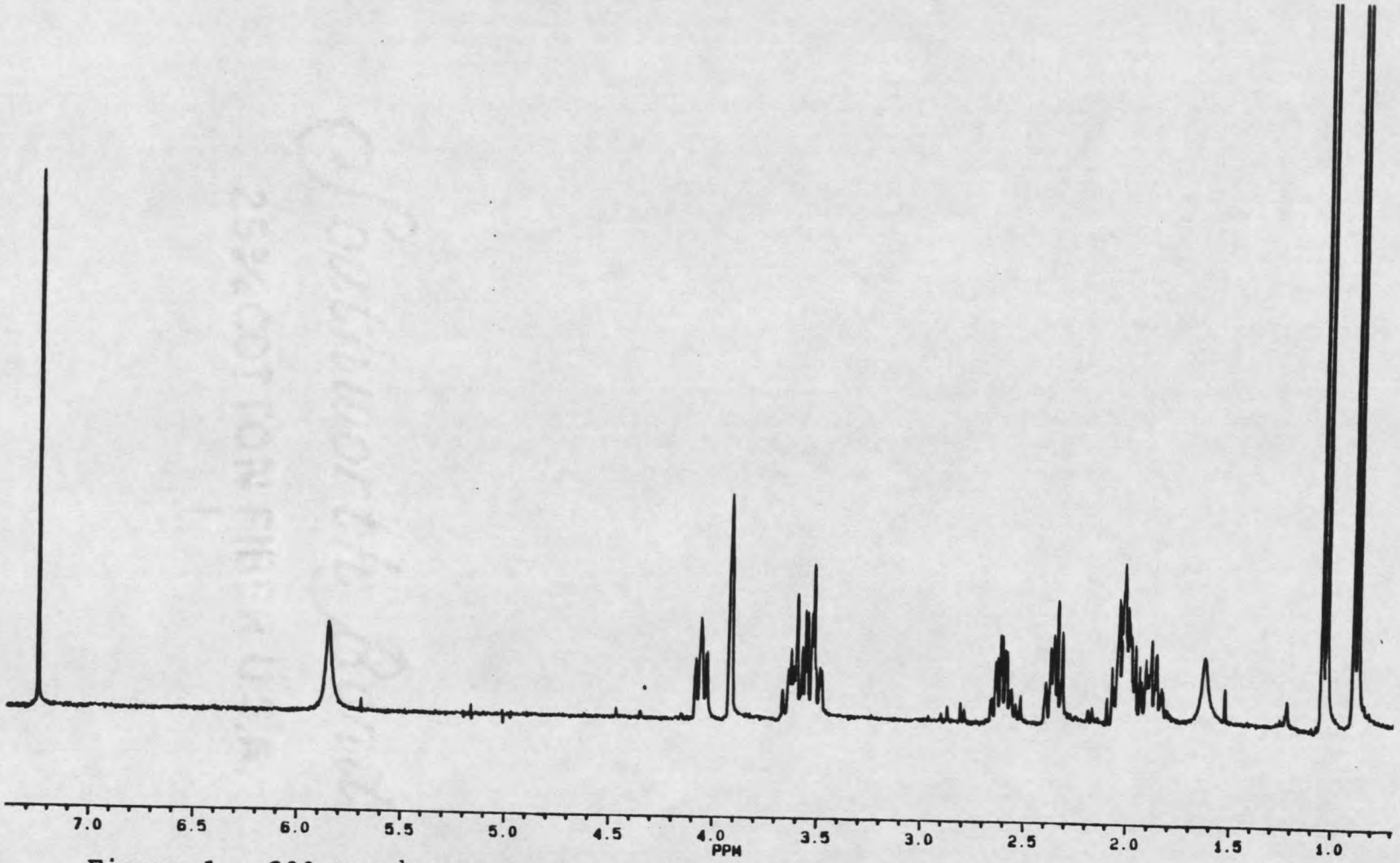


Figure 1. 300 MHz ^{1}H NMR spectrum of **7** in $CDCl_3$.

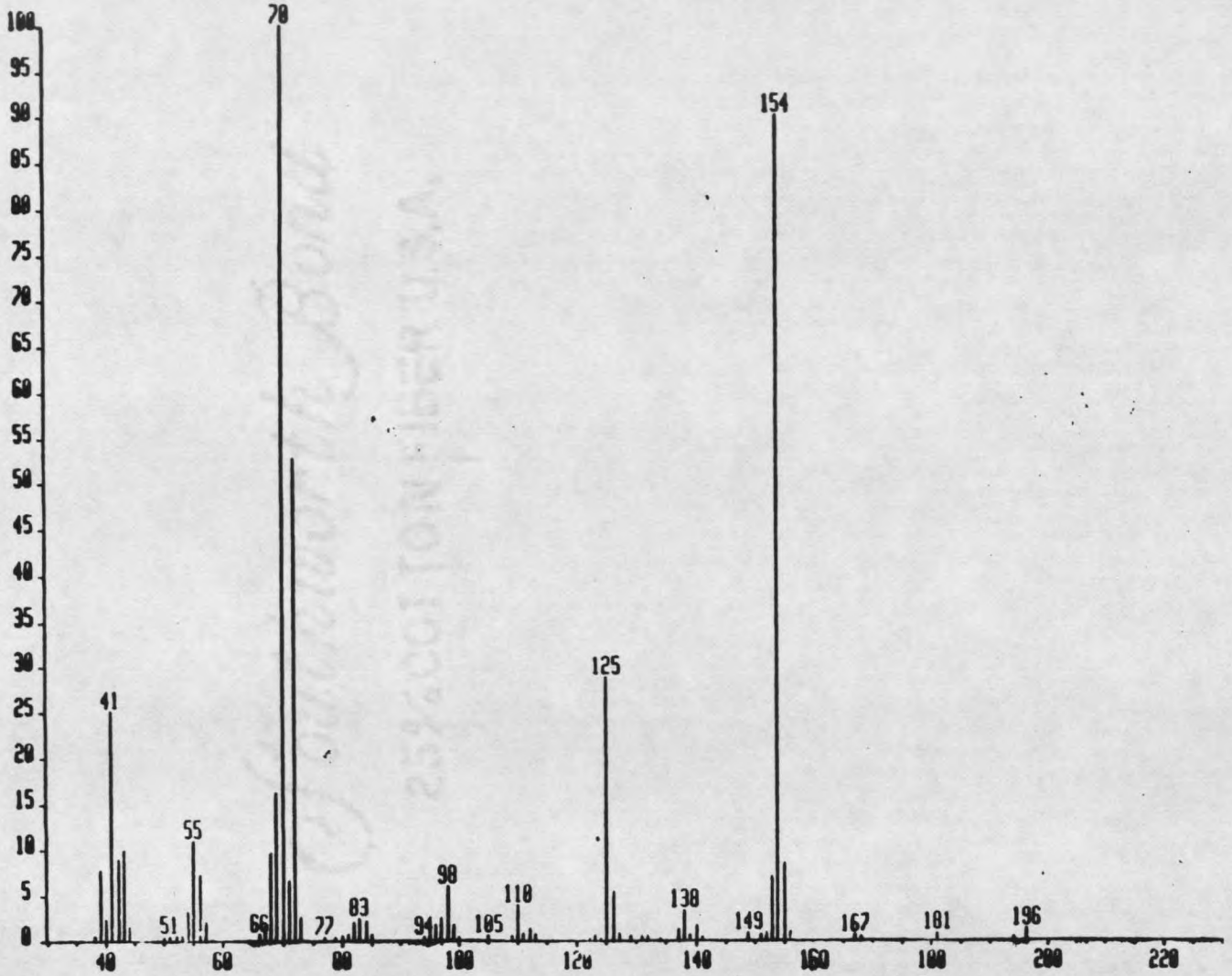


Figure 2. EI mass spectrum of 7.

moiety gave a peak at m/z 154 representing the diamidoproline structure. An optical rotation of the compound was measured at -113.7°. While this does not exactly match the literature value of -180.5° (30) (probably due to impurities and errors in weighing), it suggests the compound is the L,L-isomer.

Cyclo(L-Pro-L-Phe), 13, was also a well known diketopiperazine (31). Again, the proline moiety was recognized by the four signals at δ4.05 (1H, t), δ3.60 (2H, m), δ2.30 (2H, m) and δ2.00 (2H, m) in the ^{1}H NMR spectrum (Fig. 3). The five proton multiplet at δ7.35-7.15 indicated a monosubstituted aromatic ring. This, coupled with the methylene doublet of doublets at δ2.75 and the methine doublet of doublets at δ4.75, confirmed the phenylalanine group. The M+ peak at m/z 244, with a major fragment at m/z 153 in the mass spectrum (Fig. 4), verified the structure. Confirmation that the amino acids were indeed the L,L-isomers came, once again, through optical activity. The optical rotation for 13 was measured at -117.7°, matching the literature value of -115.6° (30).

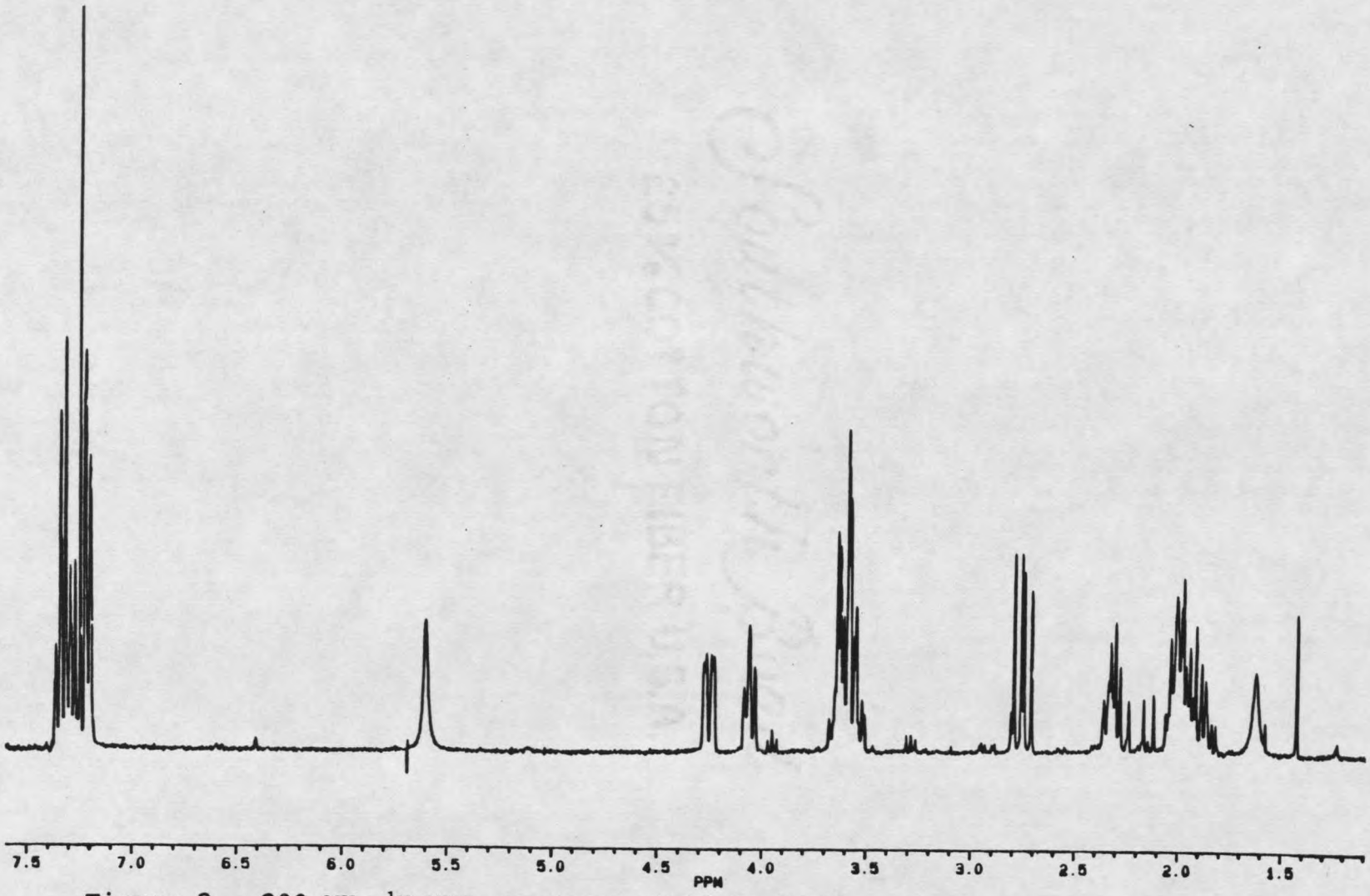


Figure 3. 300 MHz ^{1}H NMR spectrum of **13** in $CDCl_3$.

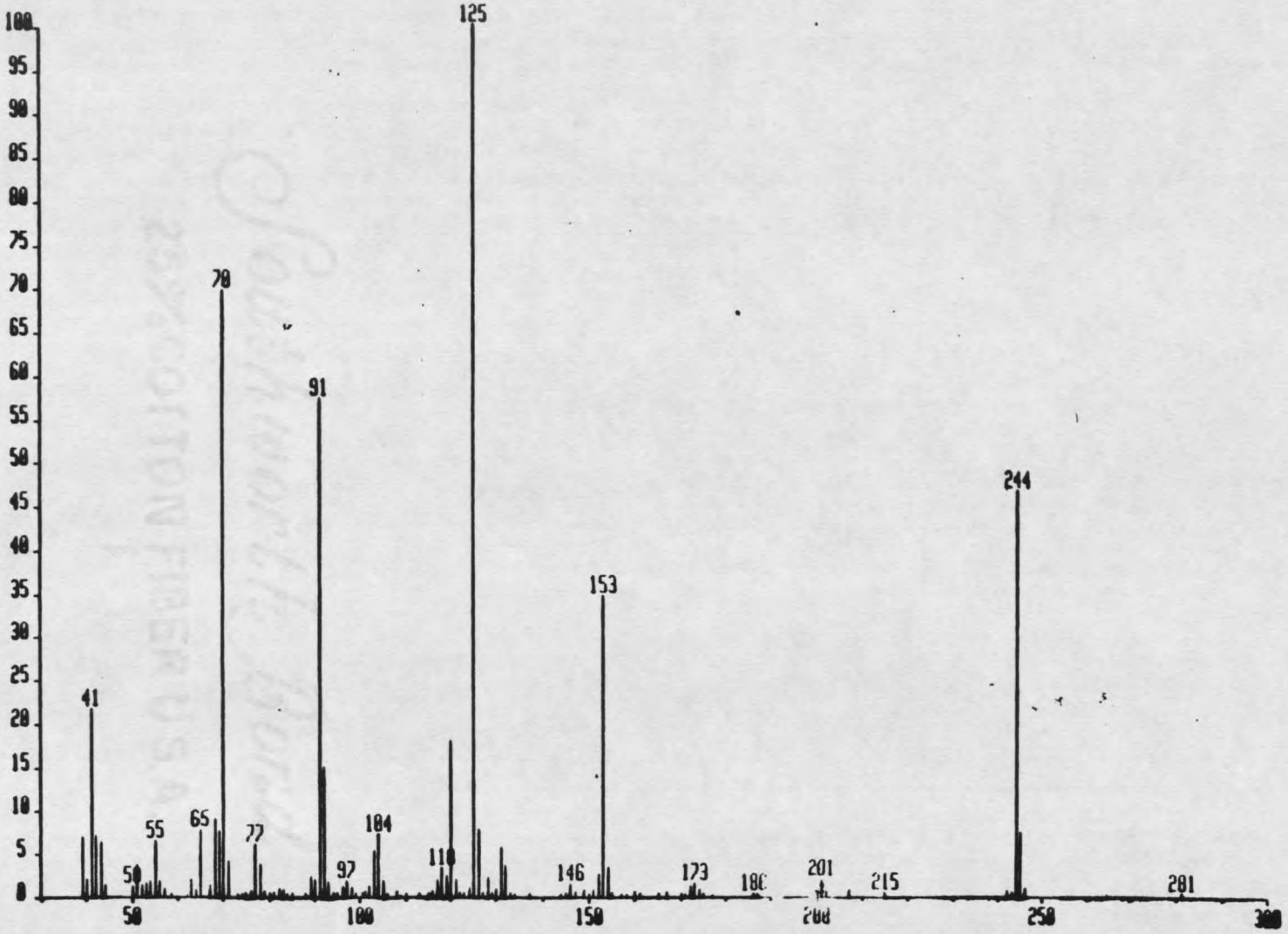
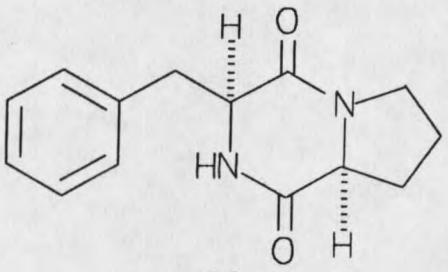


Figure 4. EI mass spectrum of 13.



13

One diketopiperazine was not immediately recognizable. The ^{1}H NMR spectrum (Fig. 5) showed proline was once again one of the amino acids with the signals at δ4.20 (1H, t), δ3.55 (2H, m), δ2.35-2.15 (2H, m) and δ2.05-1.95 (2H, m). The rest of the protons had chemical shifts that suggested they were all adjacent to heteroatoms. These were observed at δ4.7 (1H, d), δ4.5 (1H, d), δ4.4 (1H, t), δ3.45 (1H, dd) and δ3.35 (1H, dd). Also, no amide proton was present indicating that the second amino acid was probably cyclic.

A ^{13}C NMR analysis (Fig. 6) revealed nine carbons. Two of these were amide carbonyls. Four carbons were required for proline, leaving three carbons for the other amino acid. A mass spectrum (Fig. 7) gave a molecular ion at m/z 212, which required a molecular formula of $C_9H_{12}N_2O_2S$ and five sites of unsaturation. The diamido-proline accounted for four of these unsaturations,

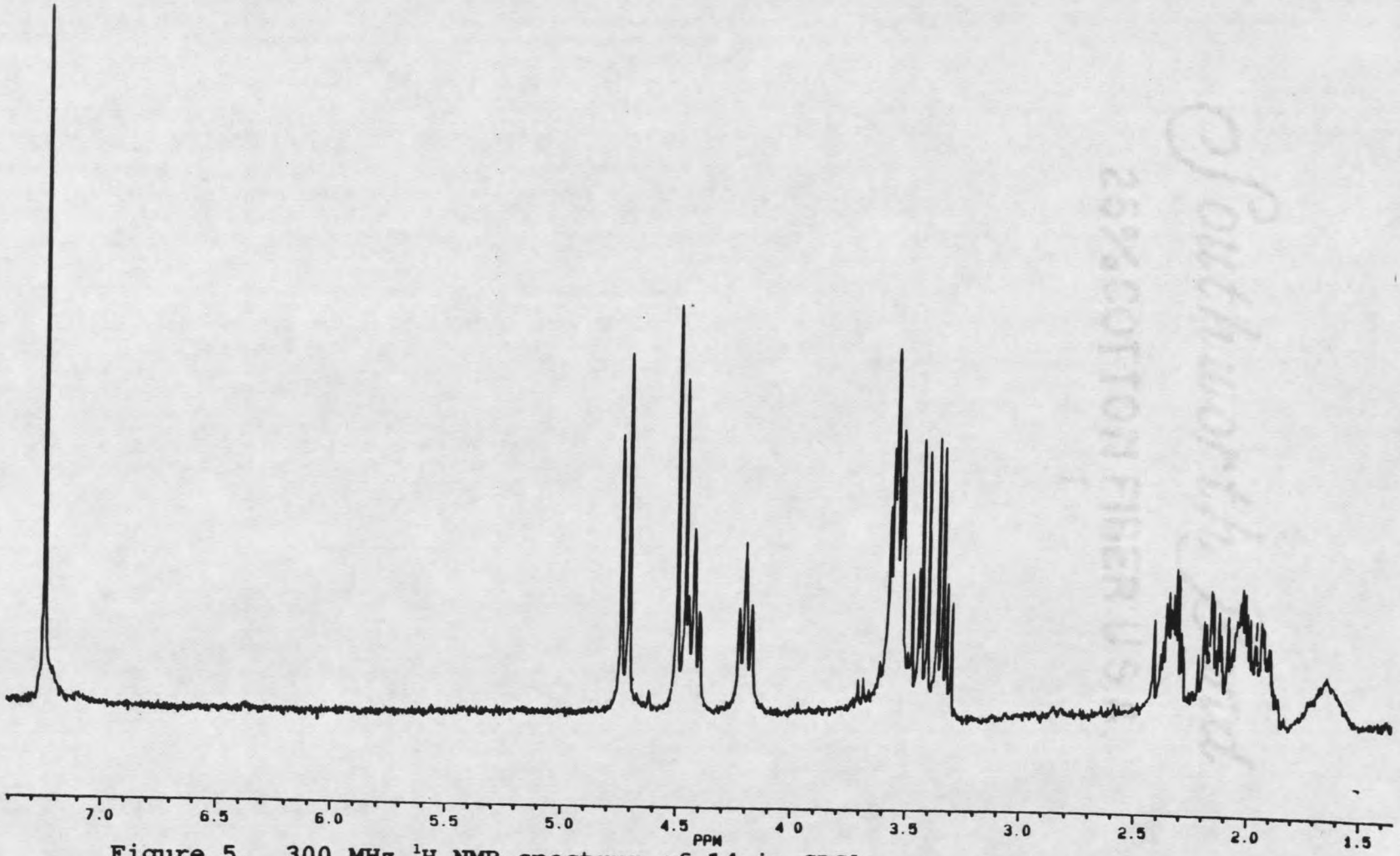


Figure 5. 300 MHz ^{1}H NMR spectrum of **14** in $CDCl_3$.

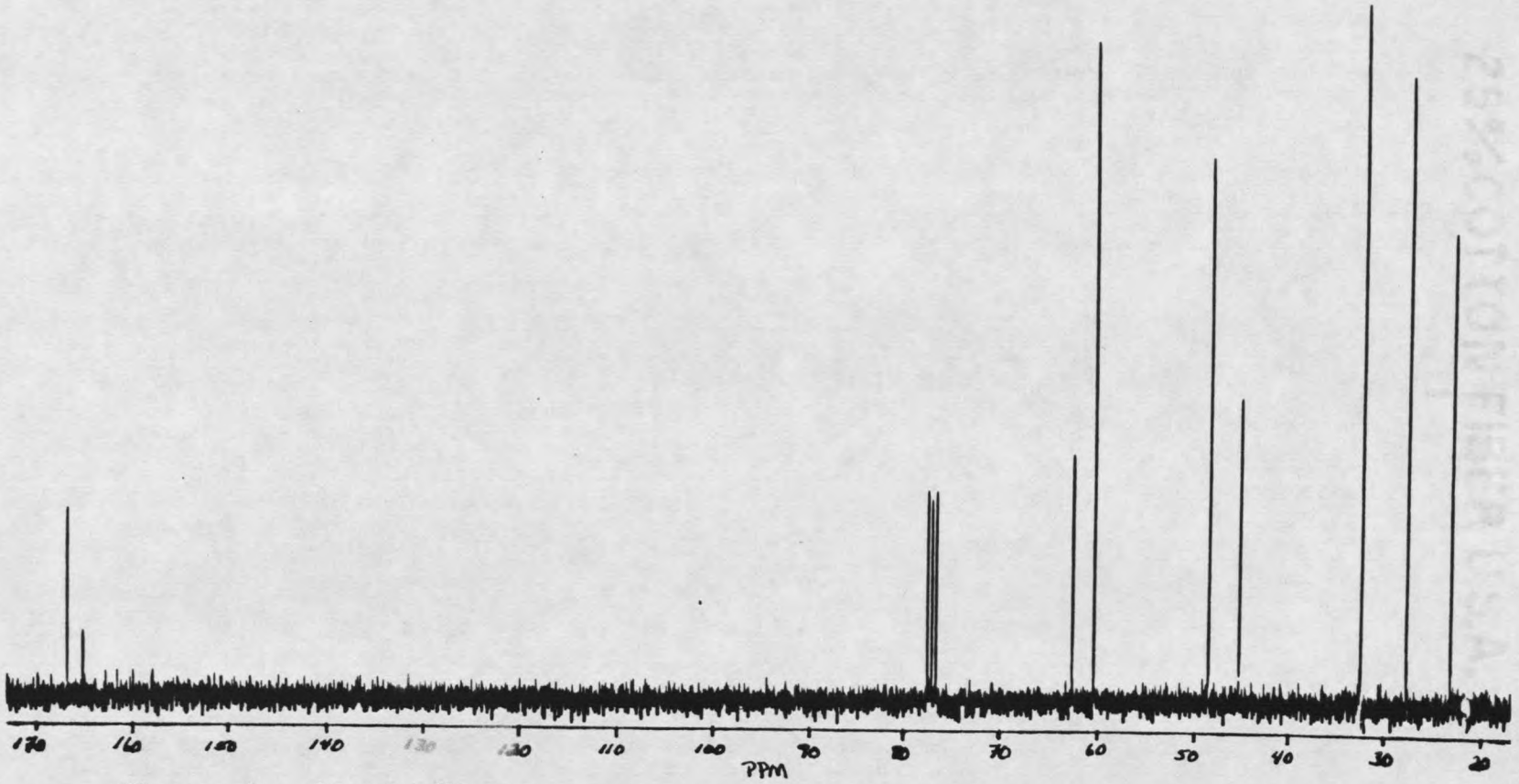


Figure 6. 75 MHz ^{13}C NMR spectrum of **14** in $CDCl_3$.

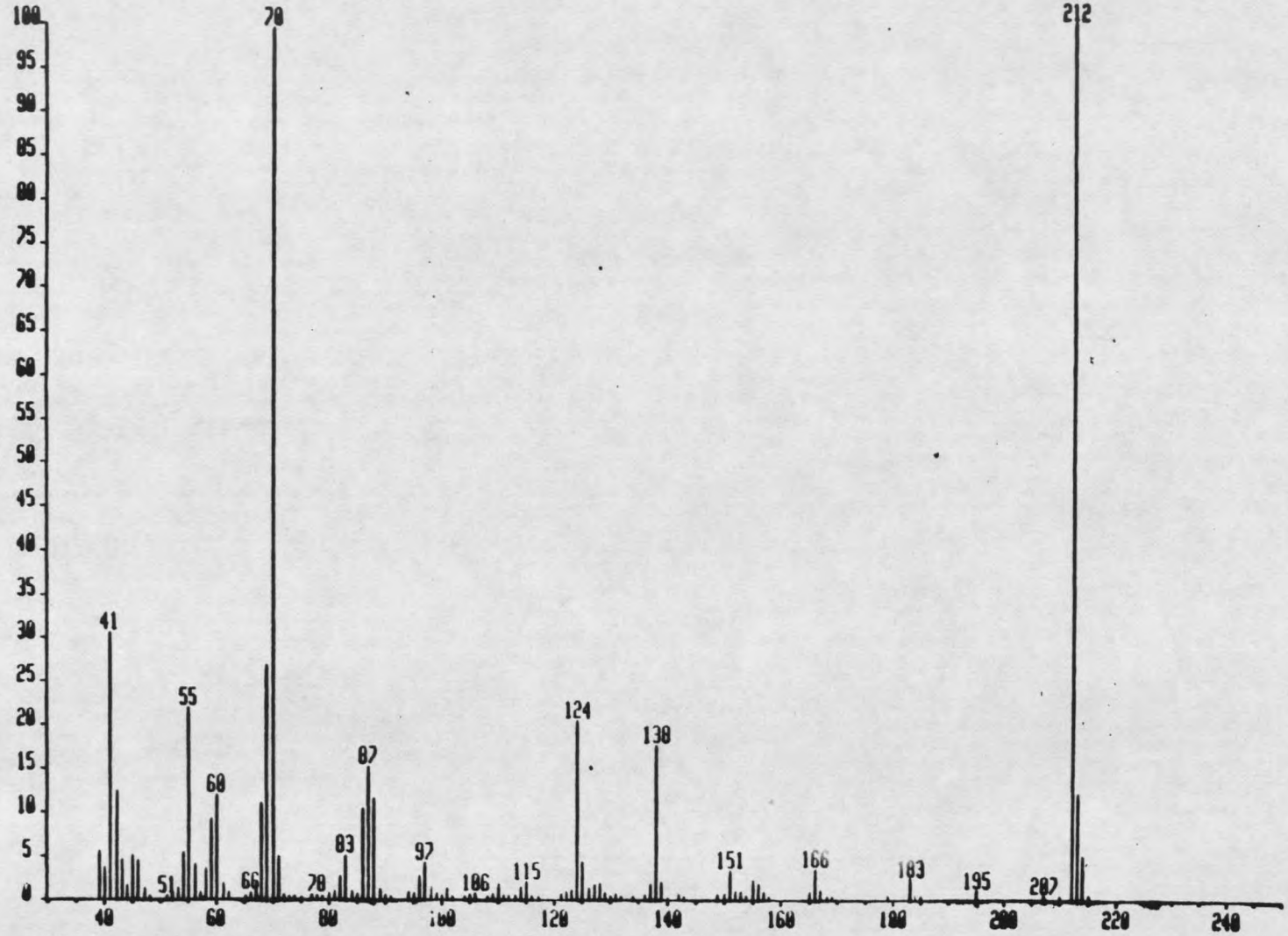
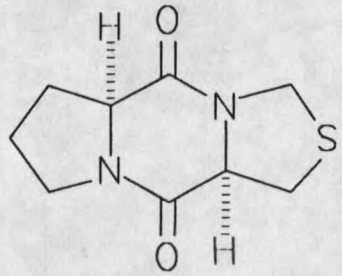


Figure 7. EI mass spectrum of 14.

confirming the idea that the second amino acid was cyclic.

A $^{1}H,^{1}H$ correlated 2D NMR (COSY) experiment (Fig. 8) revealed that the protons at δ4.7 and δ4.5 were mutually coupled. Their large J value indicated that they were geminal protons and their chemical shifts placed them between two heteroatoms, which had to be nitrogen and sulfur. The protons at δ3.45 and δ3.35 were also geminal and were coupled into the methine at δ4.4. Their chemical shifts placed them adjacent to one heteroatom. The proton at δ4.4 was the ring juncture α-methine. A HETCOR experiment (Fig. 9) allowed correlation assignments to be made between corresponding carbons and protons (Table 1). This combined spectral data gave the structure of cyclo(Pro-ThioPro). Although D-amino acids do occur in nature, they are not very common (32) and the compound was believed to be the L,L-isomer, **14**.



14

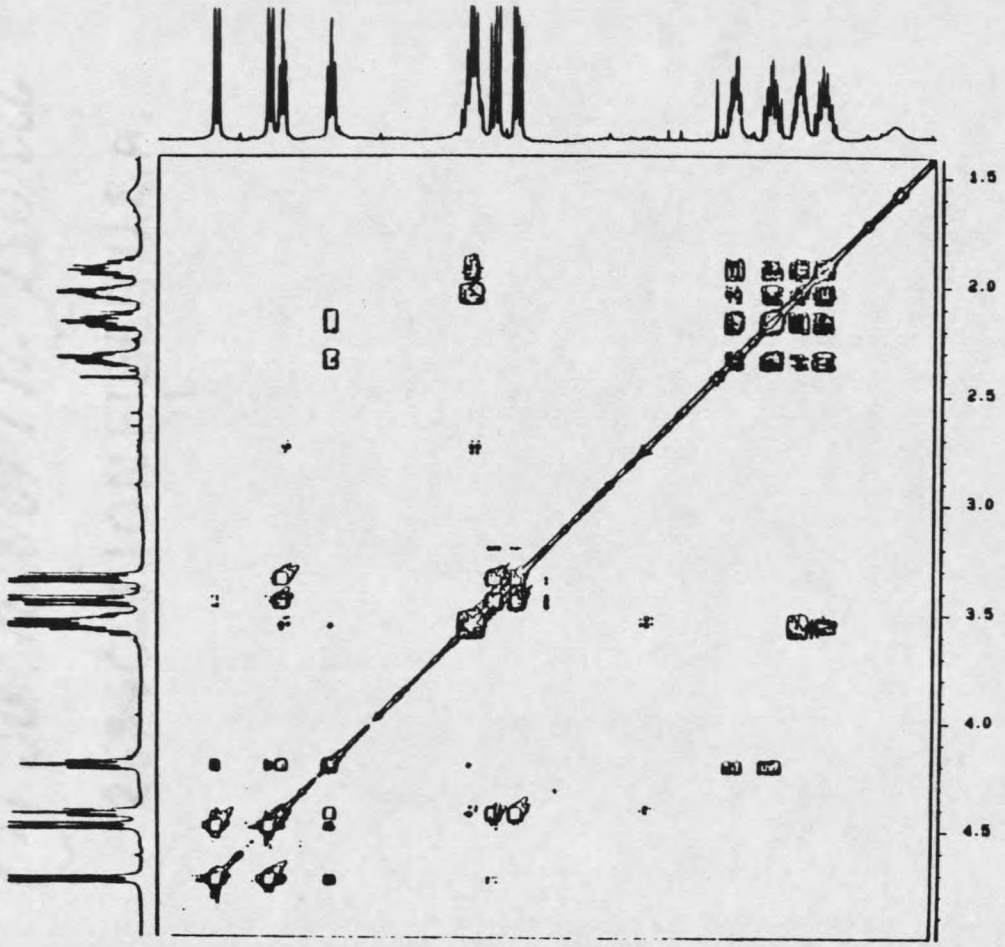


Fig. 8. 500 MHz COSY spectrum of **14** in $CDCl_3$.

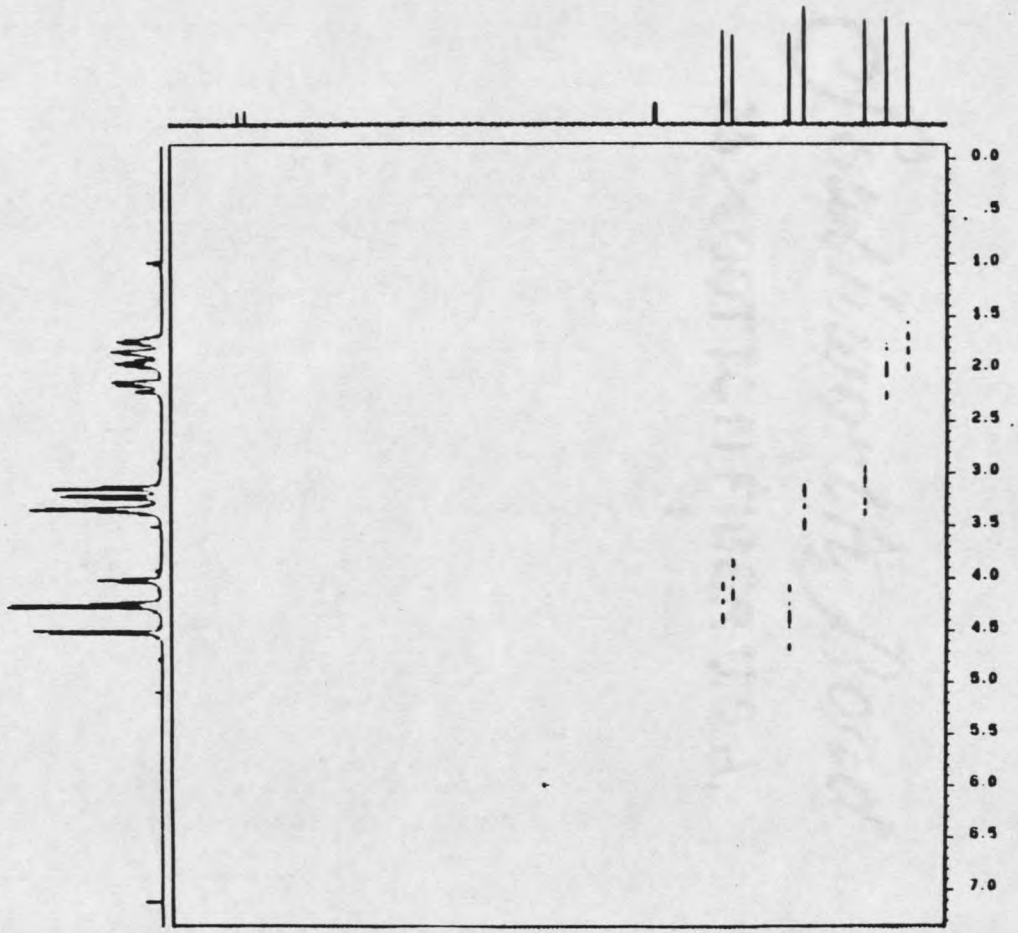


Fig. 9. 500 MHz HETCOR spectrum of **14** in $CDCl_3$.

Table 1. NMR Assignments for 14[a].

Carbon #	^{13}C	^{1}H
2	165.8[b]	- - -
3	60.5	4.2 t (J= 8 Hz)
5	164.2[b]	- - -
6	62.8	4.45 t (J= 7 Hz)
7	32.5	3.35 dd, 3.45 dd (J= 12, 7 Hz)
9	48.5	4.50 d, 4.75 d (J= 10 Hz)
10	27.8	2.15 m, 2.35 m
11	23.2	1.95 m, 2.05 m
12	45.3	3.55 m

[a] recorded in $CDCl_3$
[b] assignments are exchangeable

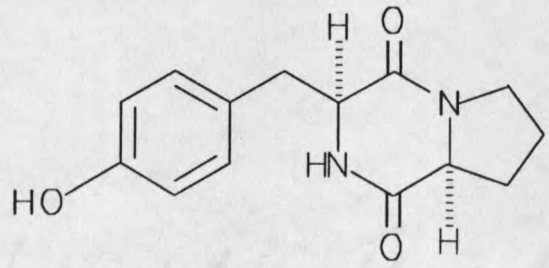
Confirmation that the compound was indeed the L,L isomer was accomplished by synthesis of cyclo(L-Pro-L-ThioPro). The synthesis was carried out using L-proline and L-thioproline and by following a simple three step procedure, the first part of which involved protecting one amino acid with BOC-ON (33). The second step reacted the protected amino acid with the methyl ester of the second amino acid, while the last step closed the diketopiperazine. The procedure was known to occur without racemization (34). The synthetic compound gave spectral data that exactly matched that of the natural product.

Optical rotations of the two compounds were in agreement, confirming the natural product as the L,L isomer.

Biological Significance and Activity of Diketopiperazines

Cyclo(L-Pro-L-ThioPro) did not seem to be in the literature. However, many other diketopiperazines containing thioproline had been synthesized and were found to have mild to strong effects as neurosedatives (35). The compound was found to be inactive in all in house bioassays at a concentration of 250 ppm. Compounds 13 and 7 were tested in the brine shrimp assay and found to be inactive also.

Earlier, a member of our research group isolated a diketopiperazine, cyclo(L-Pro-L-Tyr), 15, from the fungal plant pathogen ***Alternaria alternata*** (31). The compound was named maculosin due to its specific phytotoxic activity against spotted knapweed, ***Centaurea maculosa***. At a concentration of 10^{-3}M the compound caused weeping necrotic lesions of >4mm on knapweed leaves (31).



15

Since a diketopiperazine with thioproline had been found, it was decided to synthesize an analog of maculosin using thioproline in place in proline. The question that needed to be answered was whether or not the sulfur would change the efficacy and/or specificity of the compound.

The procedure used to synthesize cyclo(L-Tyr-L-ThioPro), **16**, was the same as that employed in the synthesis of cyclo(L-Pro-L-ThioPro). In this case, L-tyrosine was protected with the BOC-ON and the methyl ester of L-thioproline was used. Several unsuccessful attempts resulted when the synthesis was performed using L-thioproline-BOC and L-tyrosine methyl ester.

Mass spectral analysis gave a molecular ion at m/z 278 which required a molecular formula of $C_{13}H_{14}N_2O_3S$ (Fig. 10). Two major fragments were seen, one at m/z 172 for the diamidothioproline moiety and one at m/z 107 for the tyrosyl moiety. ^{1}H NMR (Fig. 11) and ^{13}C NMR spectra (Fig. 12) verified the structure based on known chemical shifts for L-tyrosine and L-thioproline. Carbon-proton correlation assignments were made based on these known chemical shifts (Table 2).

The optical rotation of the synthetic cyclo(L-Tyr-L-ThioPro) was very small, being only -2.2°. At first, this seemed to suggest that racemization had occurred during

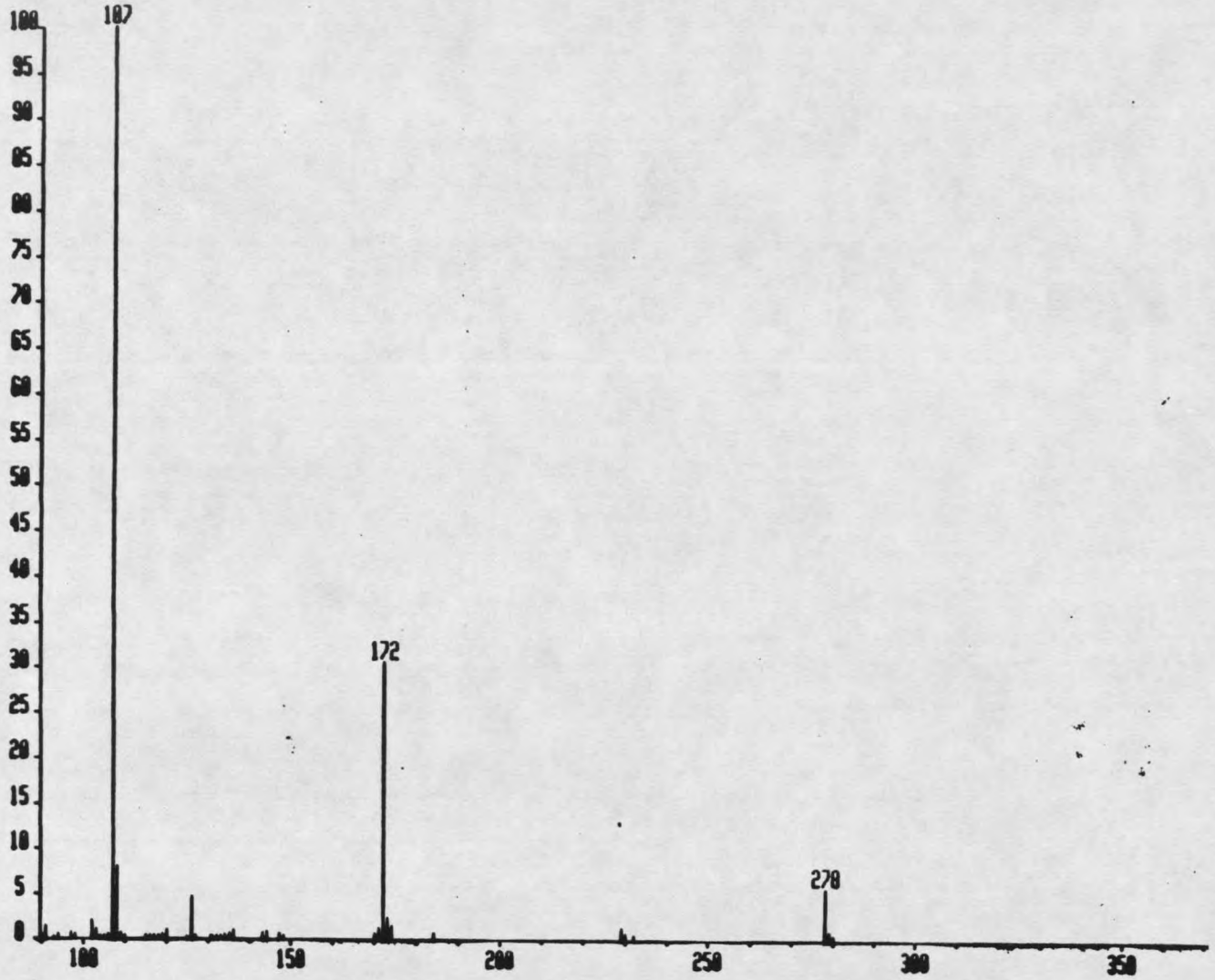


Figure 10. EI mass spectrum of 16.

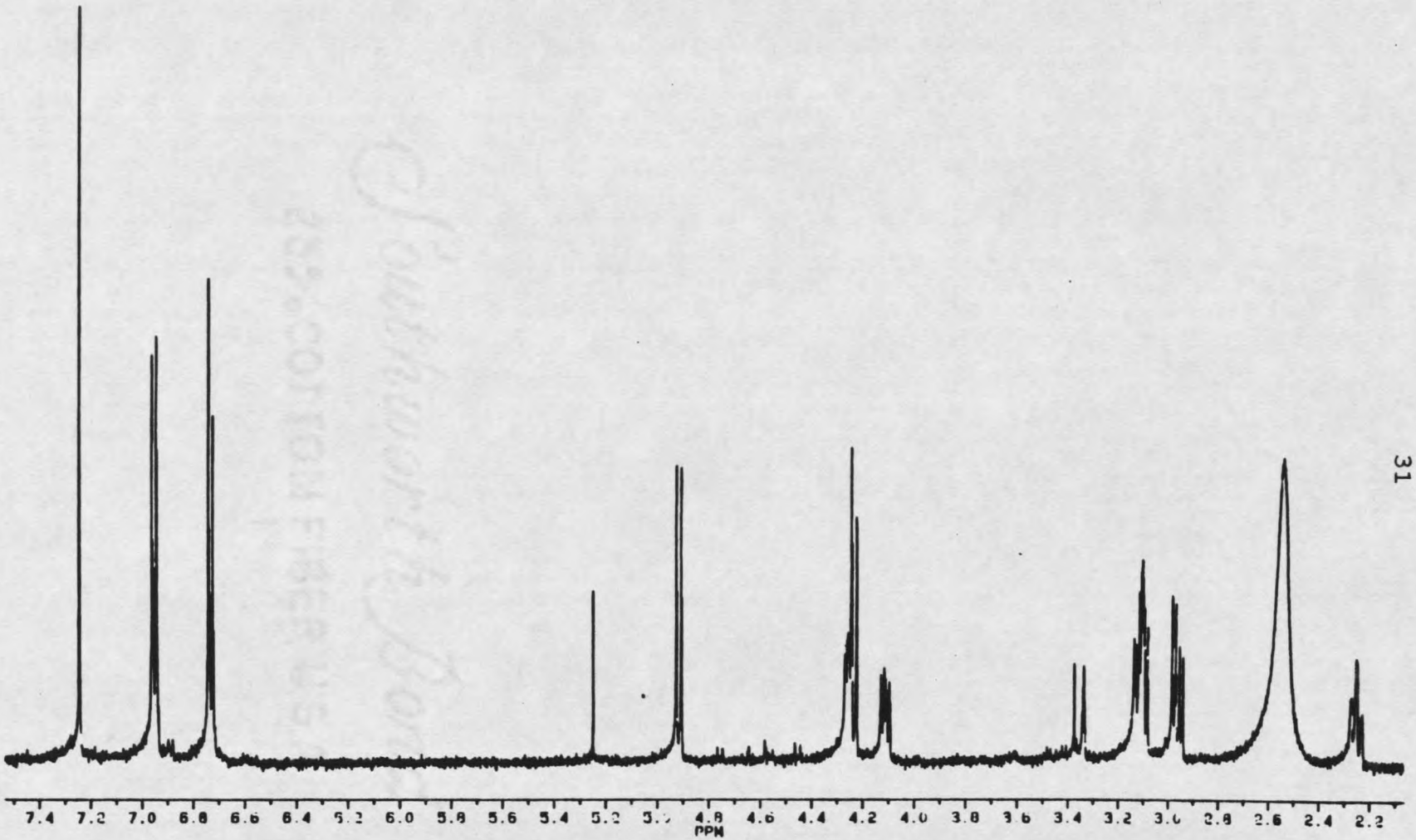


Figure 11. 500 MHz ^{1}H NMR spectrum of **16** in $CDCl_3/CD_3OD$.

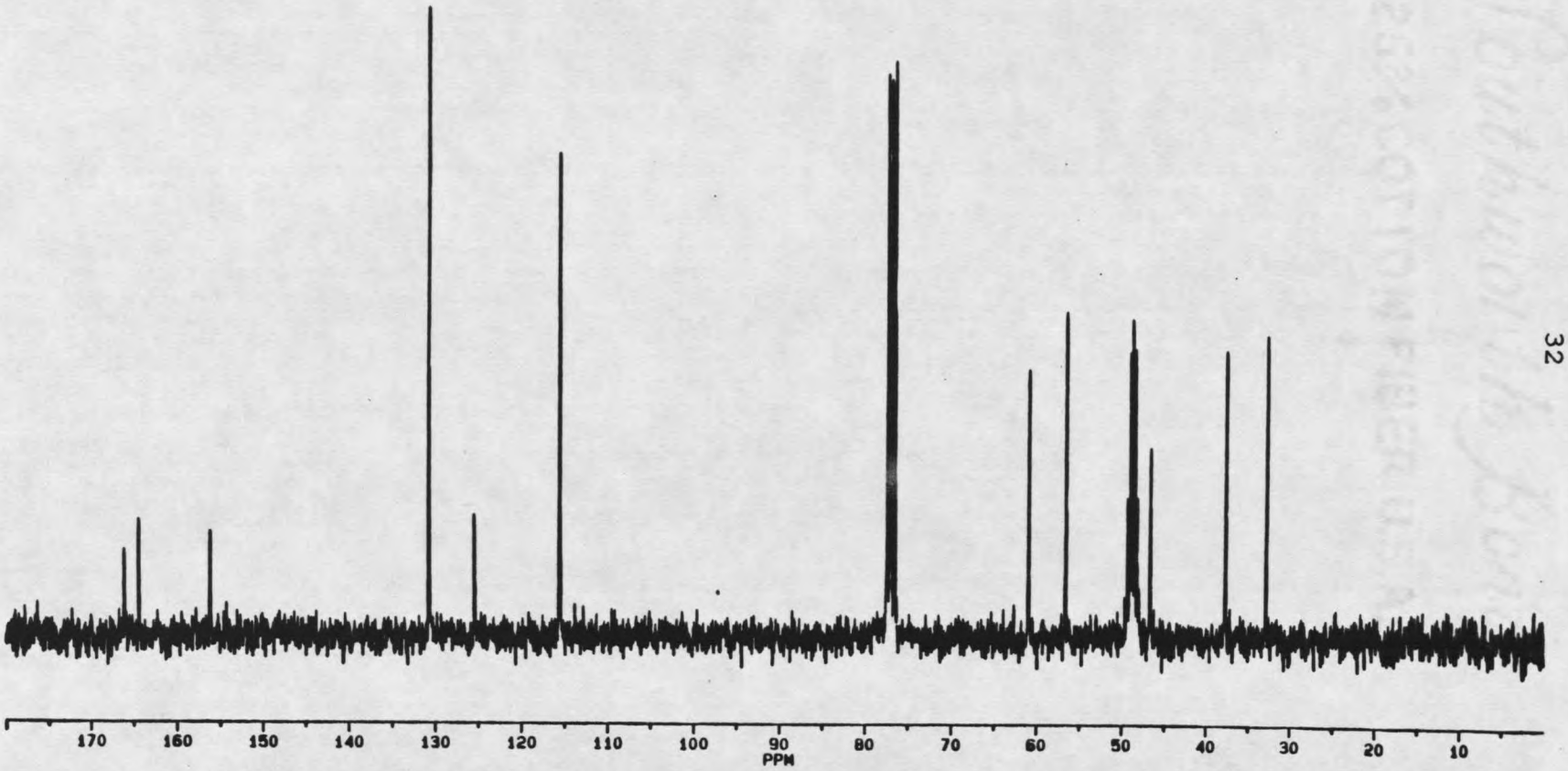
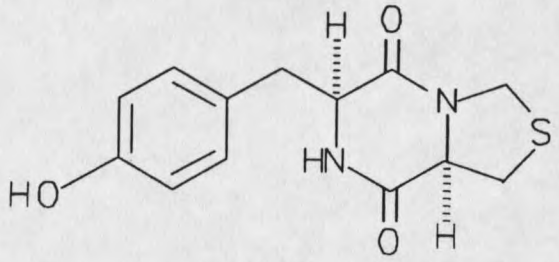


Figure 12. 75 MHz ^{13}C NMR spectrum of **16** in $CDCl_3/CD_3OD$.

the synthesis. However, the synthetic procedure is known to occur without racemization and had been used successfully before this. Also, no doubling of signals, which occurs when diastereomers are present, was seen in either the ^{1}H or ^{13}C NMR spectra.



16

Table 2. NMR Assignments for **16**[a].

Carbon #	^{13}C	^{1}H
2	166.1[b]	- - -
3	56.6[c]	4.1 m
5	164.4[b]	- - -
6	60.9[c]	4.26 t
7	32.9[d]	2.9 dd, 3.1 m
9	46.7	4.2 d, 4.9 d
10	37.7[d]	2.25 dt, 3.1 m
11	125.4	- - -
12, 16	130.7	6.9 d
13, 15	115.6	6.7 d
14	156.2	- - -

[a]recorded in $CDCl_3/CD_3OD$
[b,c,d]assignments are exchangeable

The synthetic products, **16** and **14** were then tested against spotted knapweed along side of maculosin, all at a concentration of 10^{-3}M. Both sulfur compounds were found to be inactive while maculosin showed its usual activity. The fact that the sulfur in **16** completely negates the activity was a little surprising at first. However, when one considers the size of sulfur and its lone pairs of electrons, it is not unreasonable to think that **16** could be involved in other reactions or that the sulfur prevents the molecule from "fitting" into a receptor.

Structure Elucidation of *Tedania* aromatics

The first aromatic compounds to be isolated from *T. ignis* were a series of indole derivatives. The first to be characterized was **17**. Mass spectral analysis (Fig. 13) gave a molecular ion at m/z 239, which required a molecular formula of $C_{16}H_{17}NO$ as obtained from high resolution MS. This formula required nine sites of unsaturation. The base peak at m/z 196 (M^+-43) revealed the loss of CH_3CO, while the fragments at m/z 130, 117 and 89 were indicative of a monosubstituted indole (36).

The presence of an indole was corroborated by an sharp, intense peak at 3475 cm^{-1} in the IR spectrum. The

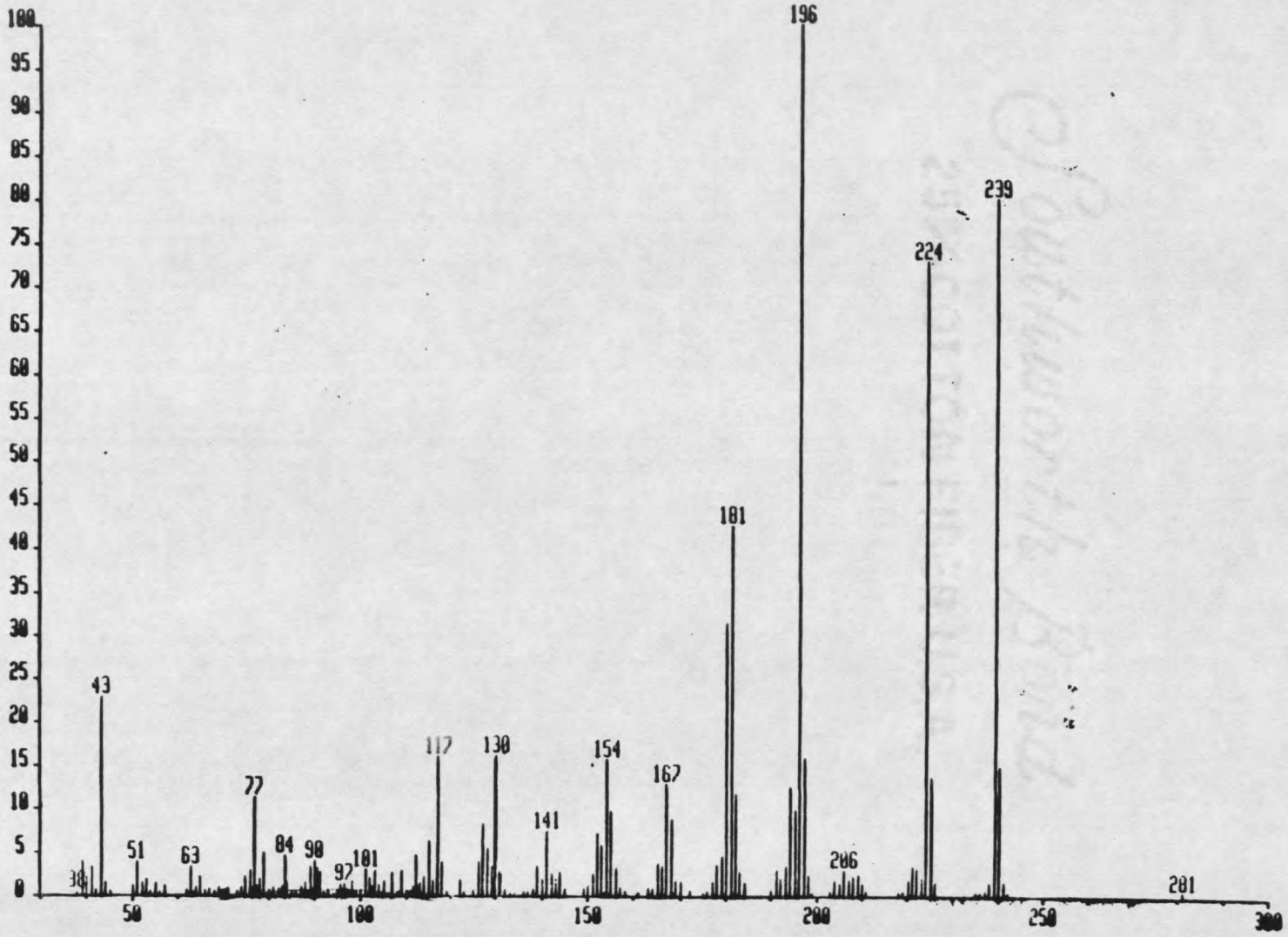


Figure 13. EI mass spectrum of 17.

IR analysis also showed a band at 1653 cm^{-1}, which was characteristic of an α,β-unsaturated ketone. The presence of a ketone was supported by the peak at δ199.4 in the ^{13}C NMR spectrum (Fig. 14).

The ^{1}H NMR spectrum (Fig. 15), recorded in d_6-acetone to spread out the aromatic signals, showed several peaks in the downfield region. The protons at δ7.4(d), 7.2(d), 7.1(t) and 6.9(t) were found to be mutually coupled by a $^{1}H,^{1}H$ COSY experiment. Their chemical shifts were representative of the protons on the benzene ring of indole. The chemical shifts of the carbons to which they were correlated, as seen in the HETCOR experiment (Fig. 16 and Table 3), supported this part of the structure.

The broad singlet at δ10.3 again signified the presence of an indole amine proton. This left the singlet proton at δ7.13 at either C-2 or C-3 of the indole. Confirmation of its position came through nOe (nuclear Overhauser effect) experiments which showed a 5.5% nOe from this proton to the amine proton at δ10.3. These results placed the proton on C-2 and left the side chain substituent on C-3. The chemical shift of this proton at δ7.13 was shifted further downfield than for the C-2 proton on an unsubstituted indole, implying the C-3 substituent was an electron withdrawing group.

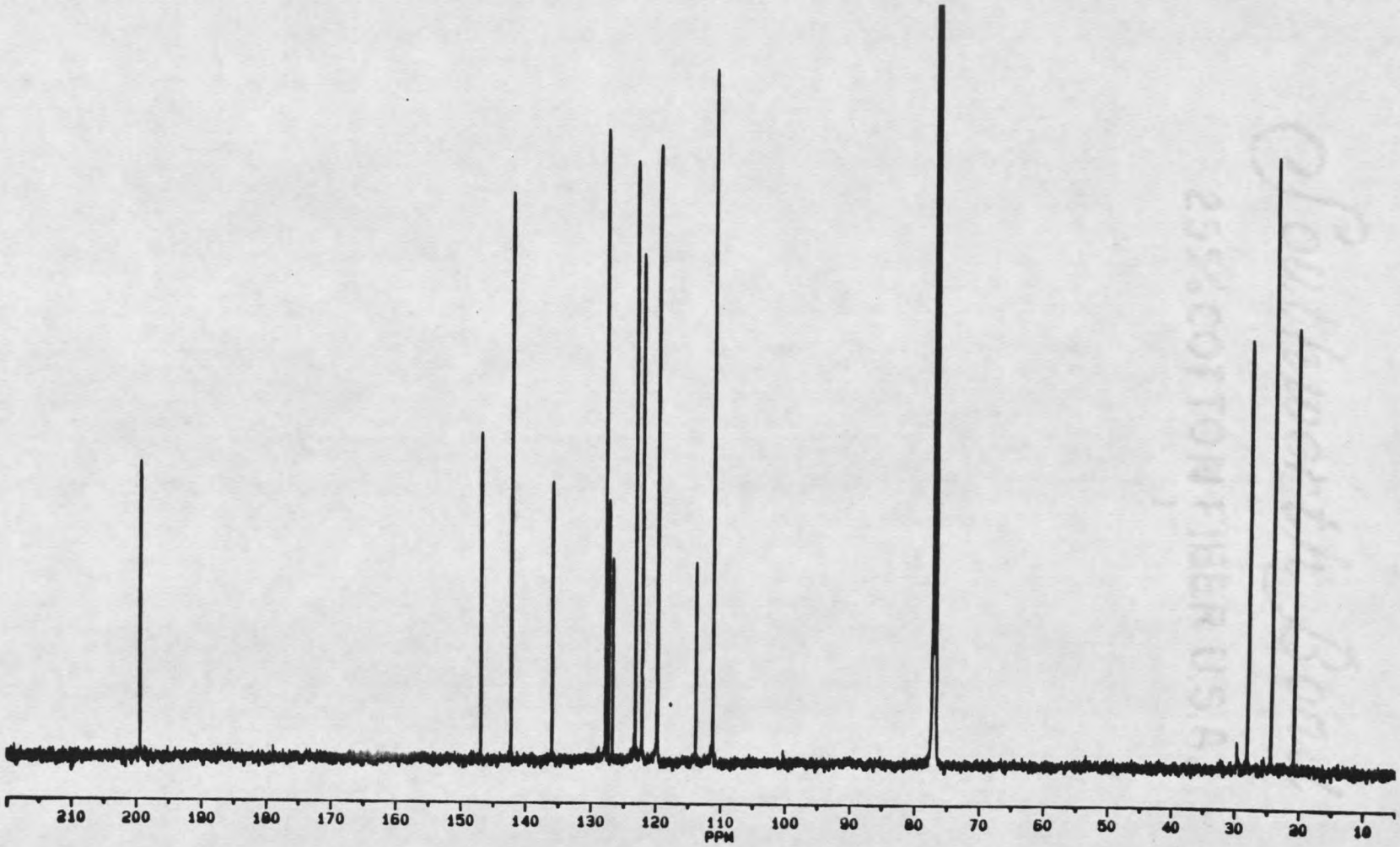


Figure 14. 125 MHz ^{13}C NMR spectrum of **17** in $CDCl_3$.

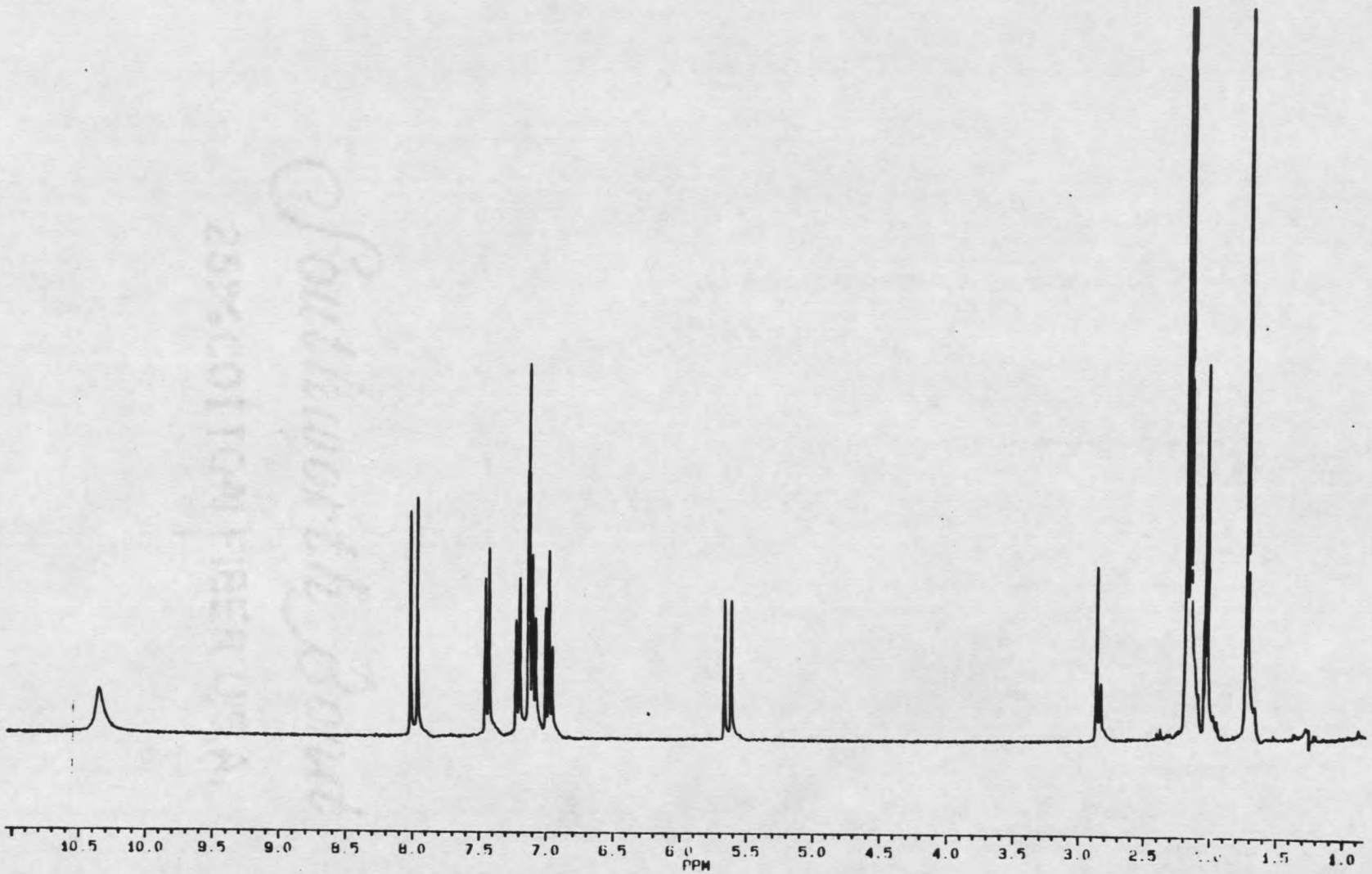
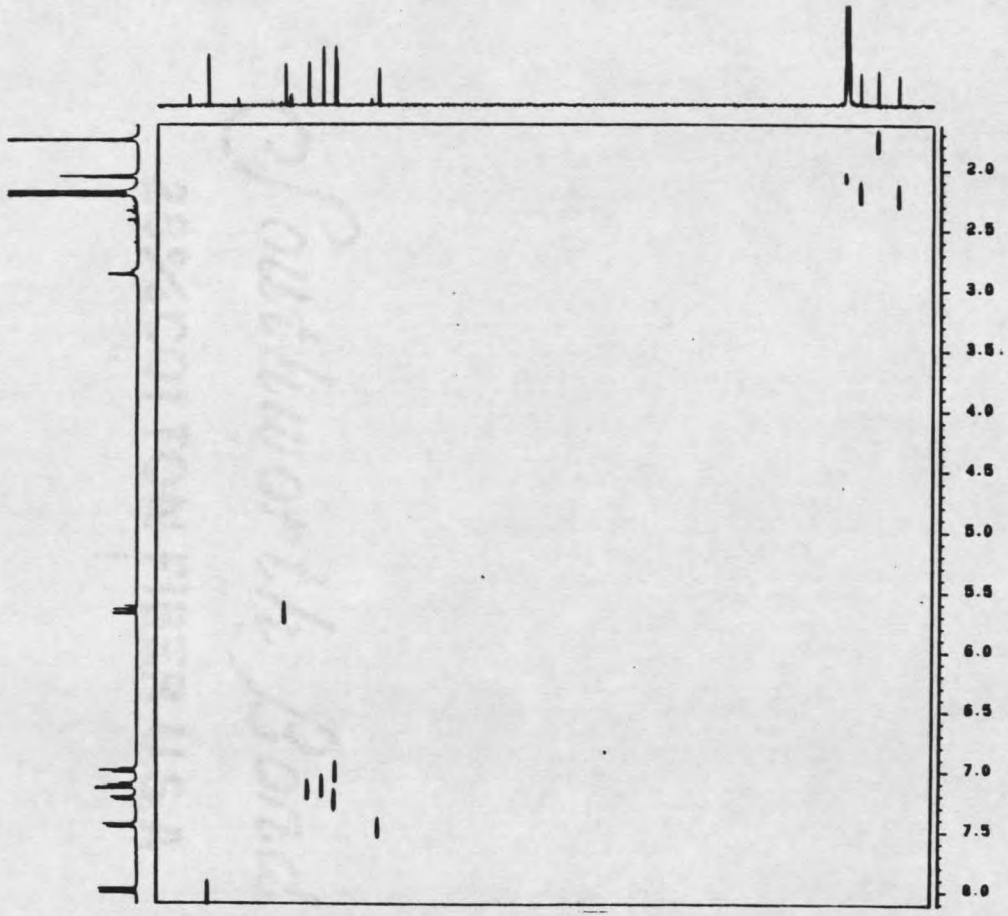


Figure 15. 300 MHz ^{1}H NMR spectrum of 17 in CD_3COCD_3.



BRUKER

0320ROXH.SMX
F1 PROJ.
0320ROH.001
F2 PROJ.
0320ROC.001
AU PROG.
XHCORR.AU
DATE 20-3-89

SI2	4096
SI1	512
SW2	17241.379
SW1	1625.488
NDO	2
WDW2	Q
WDW1	Q
SSB2	0
SSB1	0
MC2	M

PLIN ROW.

F1	171.034P
F2	34.000P

AND COLUMN.

F1	8.106P
F2	1.619P
D1	1.5000000
SI	0H
P1	12.10
DO	.0000030
P4	12.50
D3	.0035200
P3	6.30
D4	.0017600
S2	20H
RD	0.0
PW	0.0
DE	38.80
NS	96
DS	2
P9	123.00
NE	128
IN	.0001538

Figure 16. 500 MHz HETCOR spectrum of 17 in CD_3COCD_3.

Table 3. NMR Assignments for 17[a].

Carbon #	^{13}C[b]	^{1}H
2	123.1	7.13 s
3	113.8	- - -
4	119.7	7.20 d (J= 8 Hz)
5	119.6	6.90 t (J= 8, 7 Hz)
6	122.1	7.10 t (J= 8, 7 Hz)
7	111.1	7.40 d (J= 8 Hz)
8	135.9	- - -
9	126.7	- - -
10	146.8	- - -
11	127.3	- - -
12	142.2	8.00 d (J= 15.5 Hz)
13	127.7	5.60 d (J= 15.5 Hz)
14	199.4	- - -
15	28.0[c]	2.16 s
16	24.4	1.73 s
17	21.8[c]	2.19 s

[a]recorded in d_6-acetone
[b]assignments for quaternary carbons based on chemical shifts for indole (37)
[c]placement of carbons 15 and 17 is exchangeable

At this point, the presence of an indole had been fairly well established. This part structure involved C_8H_6N and 6 unsaturations leaving $C_8H_{11}O$ and 3 unsaturations in the side chain. The UV analysis of the compound showed absorption maxima that were shifted to slightly longer wavelengths than indole (38), suggesting

further conjugation of the aromatic system. Mass spectral fragmentation, as discussed earlier, indicated a CH_3CO group was present, thus leaving only C_6H_8 and 2 unsaturations.

The last two protons in the downfield region of the ^1H NMR spectrum had chemical shifts of δ8.0 and δ5.6. Both were doublets and had coupling constants of 15.5 Hz. This large J value and the large difference in their chemical shifts suggested the protons were trans to one another on a double bond that was somehow polarized. The HETCOR experiment placed the protons on carbons (Table 3) that also showed a large chemical shift difference, verifying the assumption of a polarized double bond.

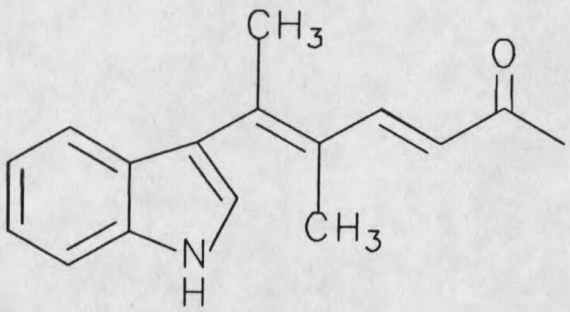
Assignments had still not been made for two carbons in the downfield region. Their shifts at δ146.8 and δ127.3 indicated another alkene which would then satify the required number of unsaturations. These carbons showed no correlation to any protons, leaving them fully substituted.

The upfield region of the ^1H NMR spectrum showed three methyl singlets at δ2.19, δ2.16 and δ1.73. One of these methyl groups was already assigned as part of the acetyl group, leaving the other two as substituents on the second double bond.

Configuration of the fully substituted double bond (whether cis or trans) was established by nOe experiments. Since the one double bond was known to be trans, two possibilities existed. If the fully substituted double bond were cis, only one methyl group would have an nOe to one of the olefinic protons. However, if the bond were trans, two nOe's should be seen (one from each methyl to one of each of the protons). As it turned out, the bond was shown to be trans. A 12% nOe was seen between the methyl at δ1.73 and the proton at δ5.6. The proton at δ8.0 showed a 9% nOe to a methyl at either δ2.19 or δ2.16, these two methyl groups being indistinguishable under irradiation.

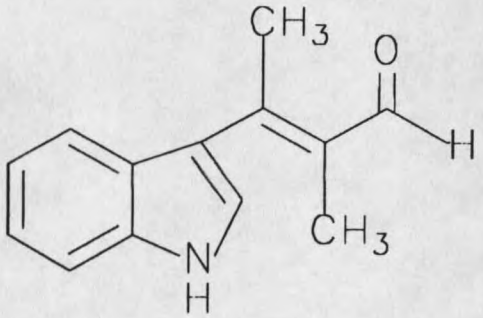
Placement of the two double bonds between the indole and the acetyl group was based on mass spectral fragmentation and chemical shift calculations (39). A major fragment in the mass spectrum, showing the loss of 43, was representative of a methyl ketone. This loss confirmed the placement of the acetyl group at the end of the side chain and supported a diene structure. Calculations showed the chemical shifts were a better fit if the unsubstituted double bond were adjacent to the acetyl group (calculated shifts = δ7.60 and δ6.30) rather than to the indole (calculated shifts = δ6.85 and δ6.65).

From these data the structure was determined to be that of 6-(indol-3-yl)-5-methylhept-3(E),5(E)-diene-2-one, **17**.



17

This indole is, however, most likely the result of an aldol condensation of the indole aldehyde **18** and acetone. Other compounds believed to be formed in this manner have been isolated from the sponges ***Dysidea etheria*** and ***Ulosa ruetzleri*** (40, 41). Neither **17** nor **18** has been reported in the literature.



18

Characterization of the rest of the indole derivatives was now much easier. Mass spectral analysis of compound **19** (Fig. 17) showed a molecular ion at m/z 185 which required a molecular formula of $C_{12}H_{11}NO$. Eight sites of unsaturation were needed. Mass spectral fragments at m/z 115 and 89 suggested an indole was again present. An IR spectrum further supported this with an sharp, intense peak at 3466 cm^{-1}.

The IR spectrum also showed a peak at 1643 cm^{-1} characteristic of an α,β-unsaturated ketone. The presence of a ketone was confirmed by a peak at δ198.7 in the ^{13}C NMR spectrum (Fig. 18).

A ^{1}H NMR analysis (Fig. 19) showed four protons whose multiplicities and chemical shifts were representative of the ortho disubstituted benzene ring of indole. These were observed at δ7.95 (1H, d), δ7.5 (1H, dd), δ7.2 (2H, m). The chemical shifts of the downfield carbons helped support the indole structure (see Table 4). Also, an amine proton was present in the ^{1}H NMR spectrum as a broad singlet at δ8.6.

The proton at δ7.88 again had to be positioned at C-2 or C-3 of indole. It appeared as a doublet with a small coupling (J = 2 Hz), implying it was coupled to the indole amine proton and thus, placing it at C-2 (42). The

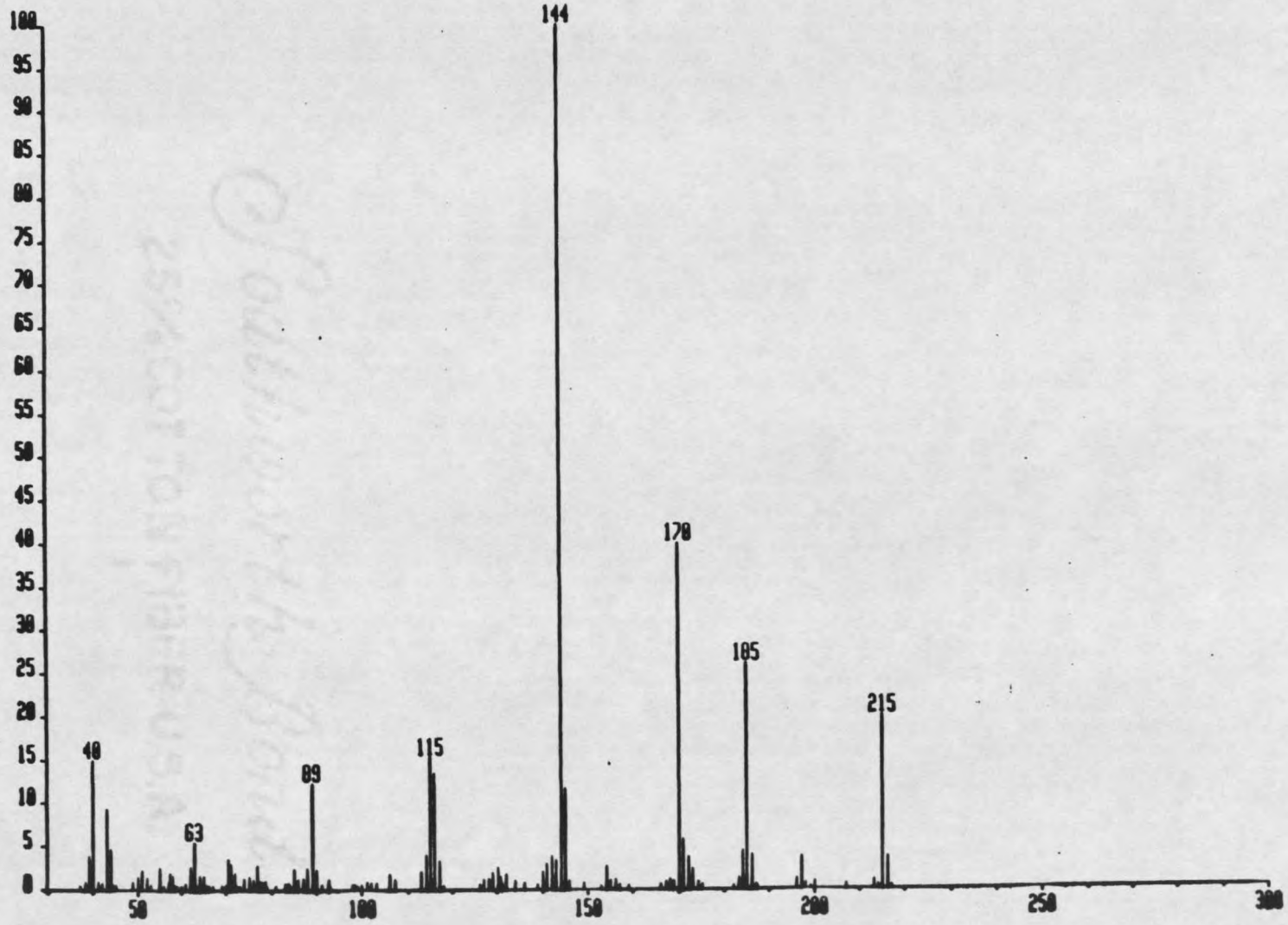


Figure 17. EI mass spectrum of 19.

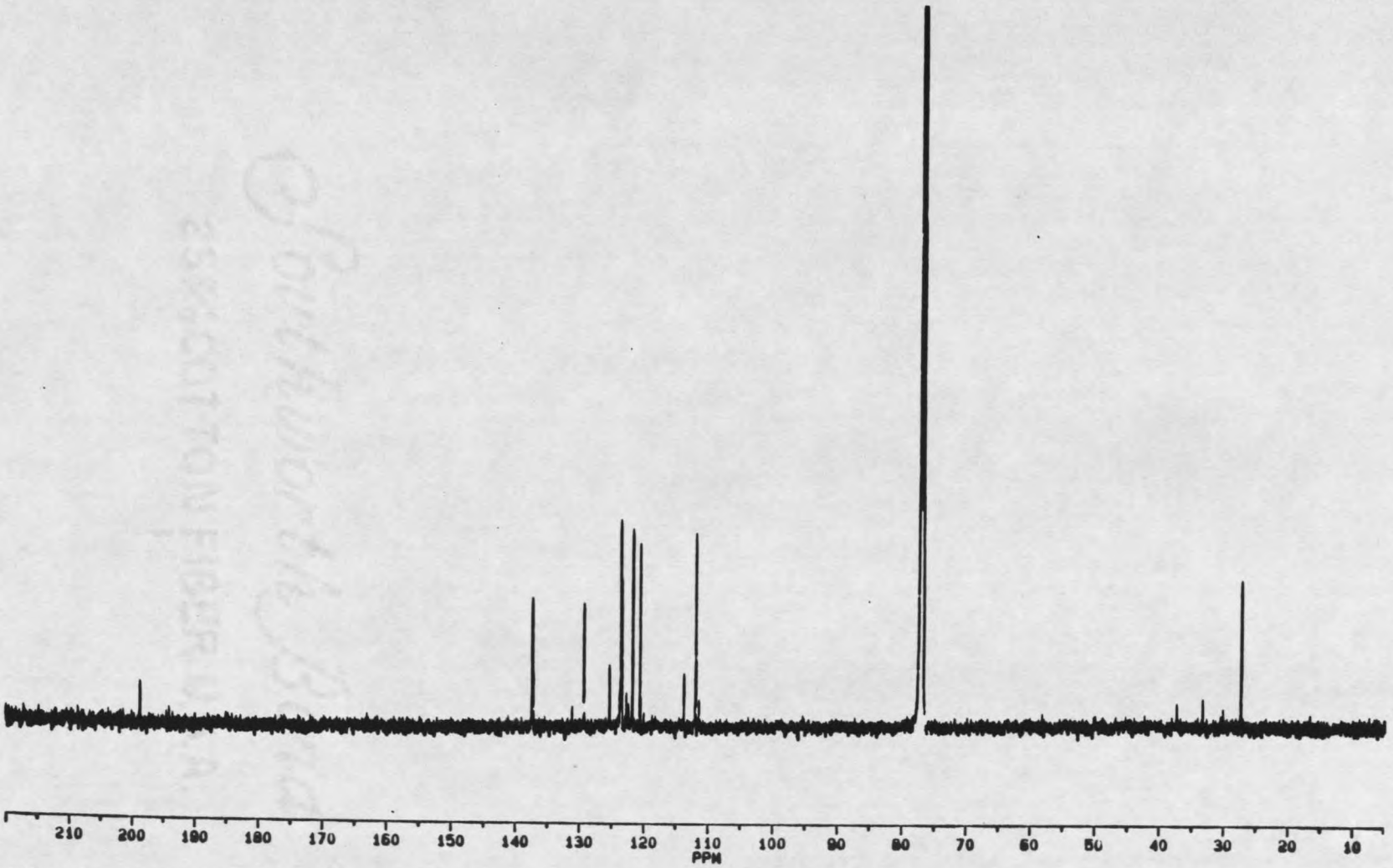


Figure 18. 125 MHz ^{13}C NMR spectrum of **19** in $CDCl_3$.

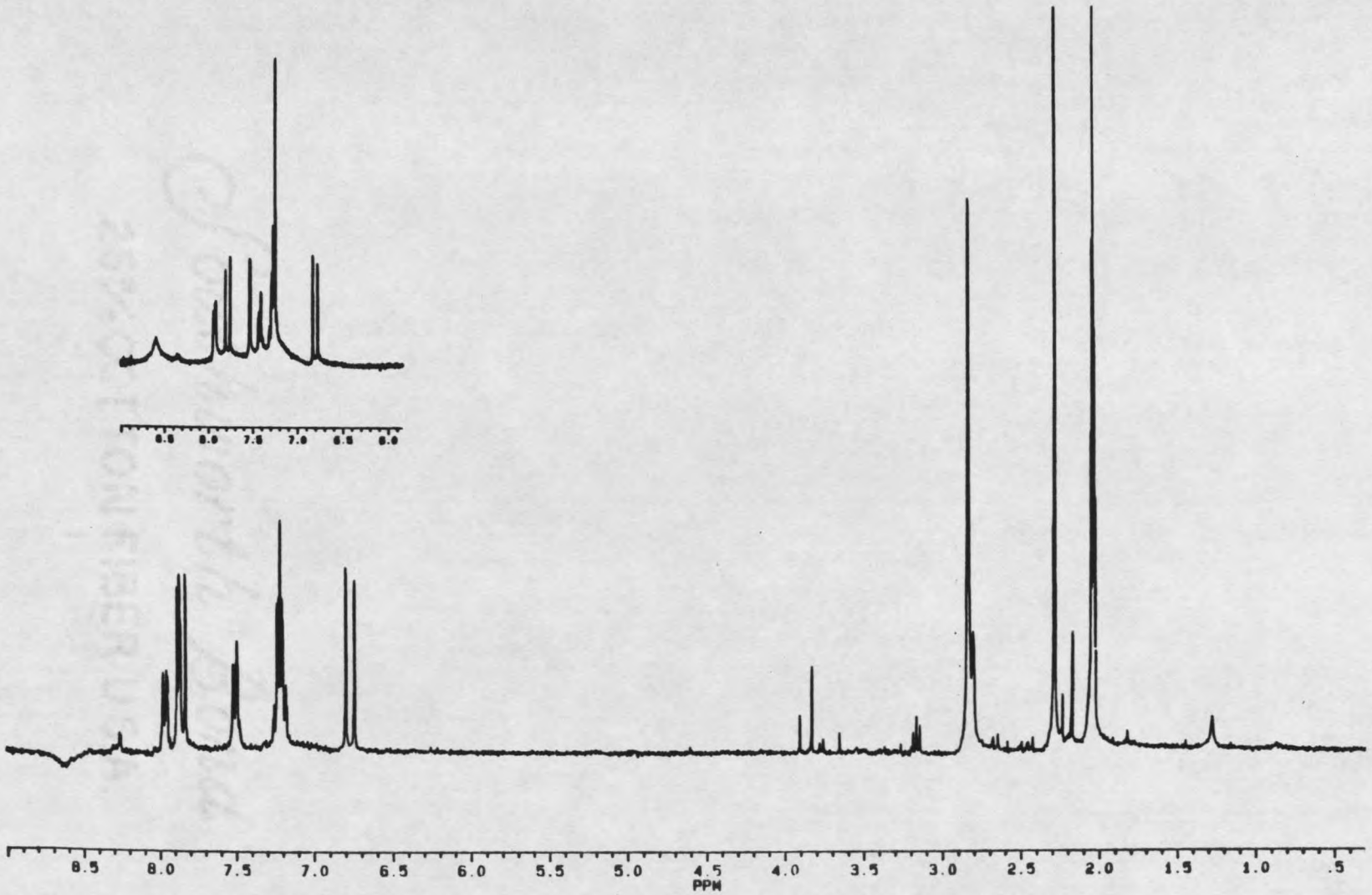
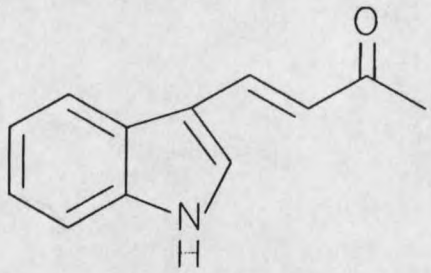


Figure 19. 300 MHz ^{1}H NMR spectrum of **19** in d_6-acetone. Inset shows spectrum in $CDCl_3$.

chemical shift indicated that the C-3 substituent was an electron withdrawing group.

The indole represented C_8H_6N and 6 unsaturations, while the ketone accounted for CO and 1 unsaturation. This left C_3H_5 and 1 unsaturation for the rest of the molecule.

The protons at δ7.85 and δ6.75 were both doublets and coupled by 16 Hz. Their large J value and large difference in chemical shift again suggested a polarized trans double bond. Chemical shift calculations placed the double bond between the indole and the ketone (39). The methyl at δ2.3 then finished the structure which was determined to be 4-(indol-3-yl)but-3(E)-en-2-one, **19**.



19

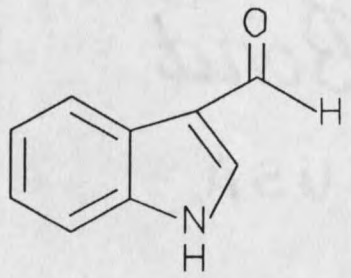
This compound was synthesized by Nonnenmacher and co-workers (43) and their ^1H NMR data confirmed the side chain structure of the natural product. They found the α

Table 4. NMR Assignments for 19, 21 and 23[a,b]

Carbon #	19		21		23	
	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H
2	123.5	7.88 d	124.4	7.19 d	134.0	8.10 s
3	113.7	- - -	112.1	- - -	114.8	- - -
4	121.7	7.50 dd	119.6	7.30 d	123.3	7.40 dd
5	120.5	7.20 m	119.3	7.00 m	122.6	7.20 m
6	123.4	7.20 m	122.3	7.00 m	124.4	7.20 m
7	111.8	7.95 d	112.2	7.50 dd	112.9	8.20 dd
8	137.2	- - -	137.8	- - -	138.2	- - -
9	125.3	- - -	128.3	- - -	126.9	- - -
10	137.3	7.85 d	29.0	3.70 dd	196.0	- - -
11	129.2	6.75 d	147.5	7.00 m	66.3	4.70 s
12	198.7	- - -	132.1	6.10 d		
13	27.4	2.30 s	198.1	- - -		
14			26.8	2.16 s		

[a] recorded in d_6-acetone
[b] assignments based on known chemical shifts for indole

and β protons at δ6.7 and 7.9, respectively, with both having coupling constants of 16 Hz. The methyl group appeared at δ2.35 as a singlet. This indole is probably an artefact formed through an aldol condensation of indole 3-carboxaldehyde, **20**, and acetone.



20

Data for **21** again pointed to an indole derivative. Mass spectral analysis gave a molecular ion at m/z 199 (Fig. 20) which required a molecular formula of $C_{13}H_{13}NO$ with eight sites of unsaturation. Mass spectral fragmentation showed a M^+-43 peak at m/z 156, which was characteristic of a methyl ketone. Peaks at m/z 130, 117 and 89 again typified fragmentation of an indole. Infrared and ^1H NMR spectra (Fig. 21) supported this with a sharp, intense peak at 3481 cm^{-1} and a broad amine proton singlet at δ10.1, respectively.

The presence of a ketone was confirmed by the peak at

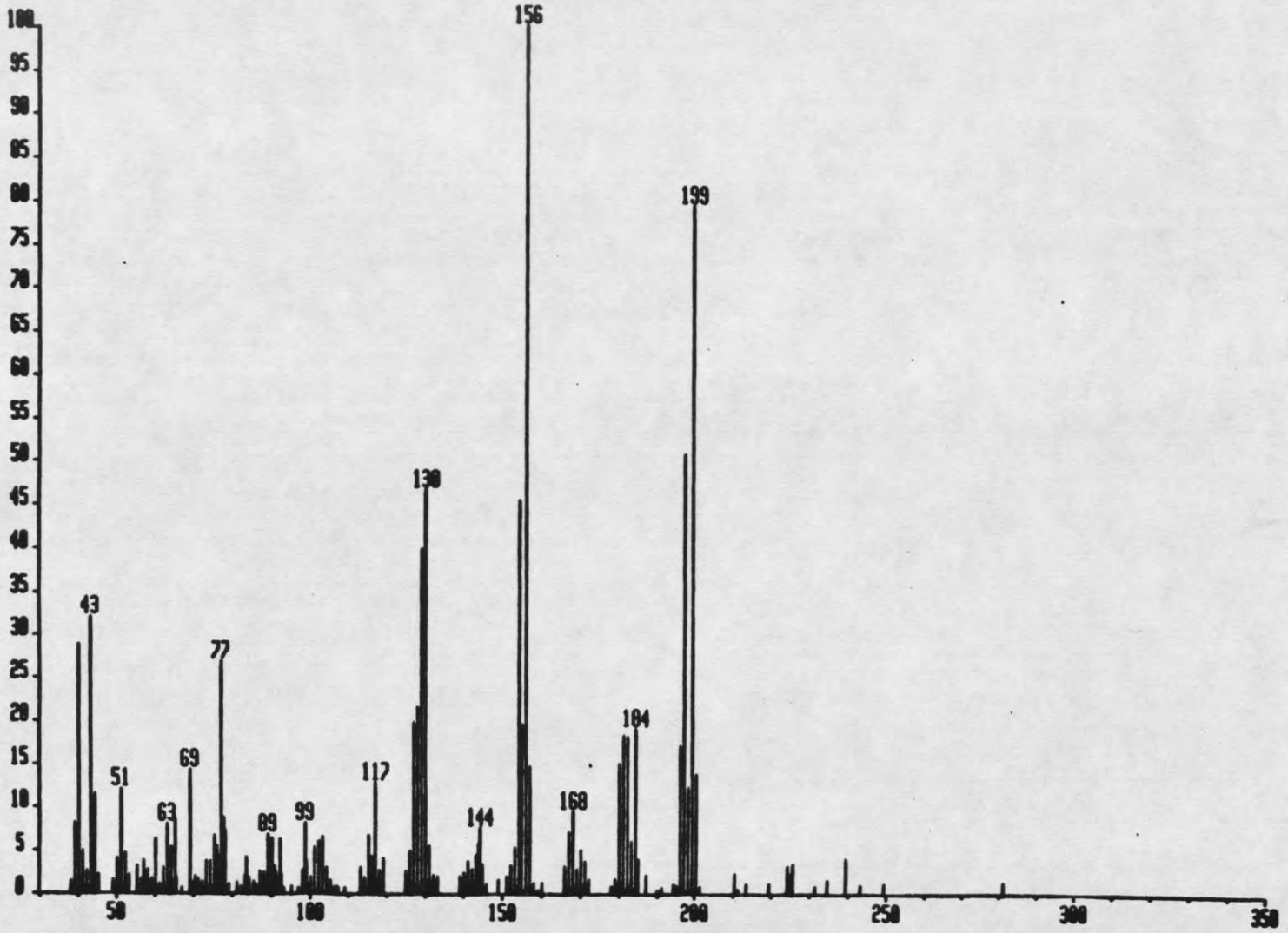


Figure 20. EI mass spectrum of 21.

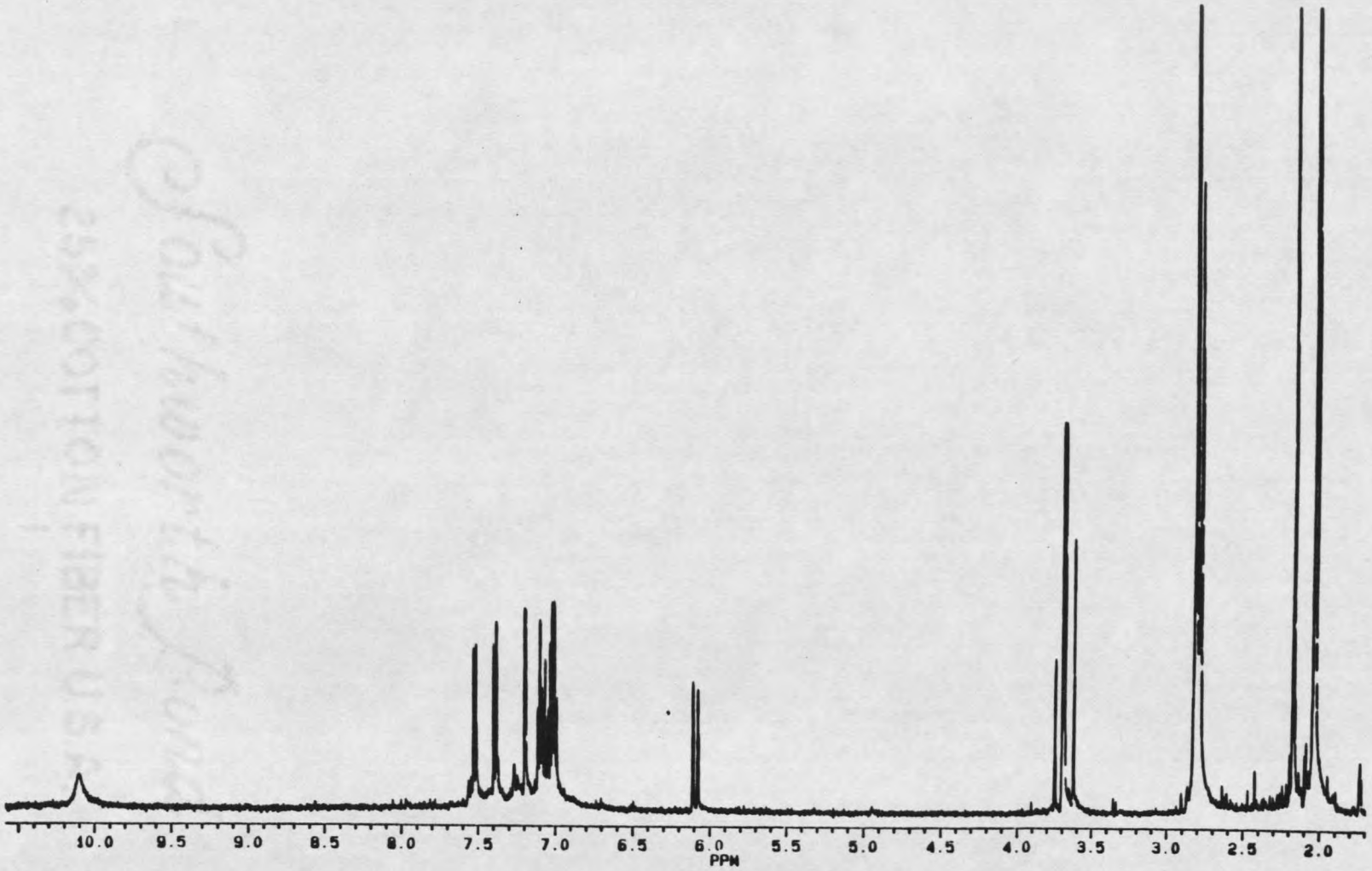


Figure 21. 500 MHz ^{1}H NMR spectrum of 21 in d_6-acetone.

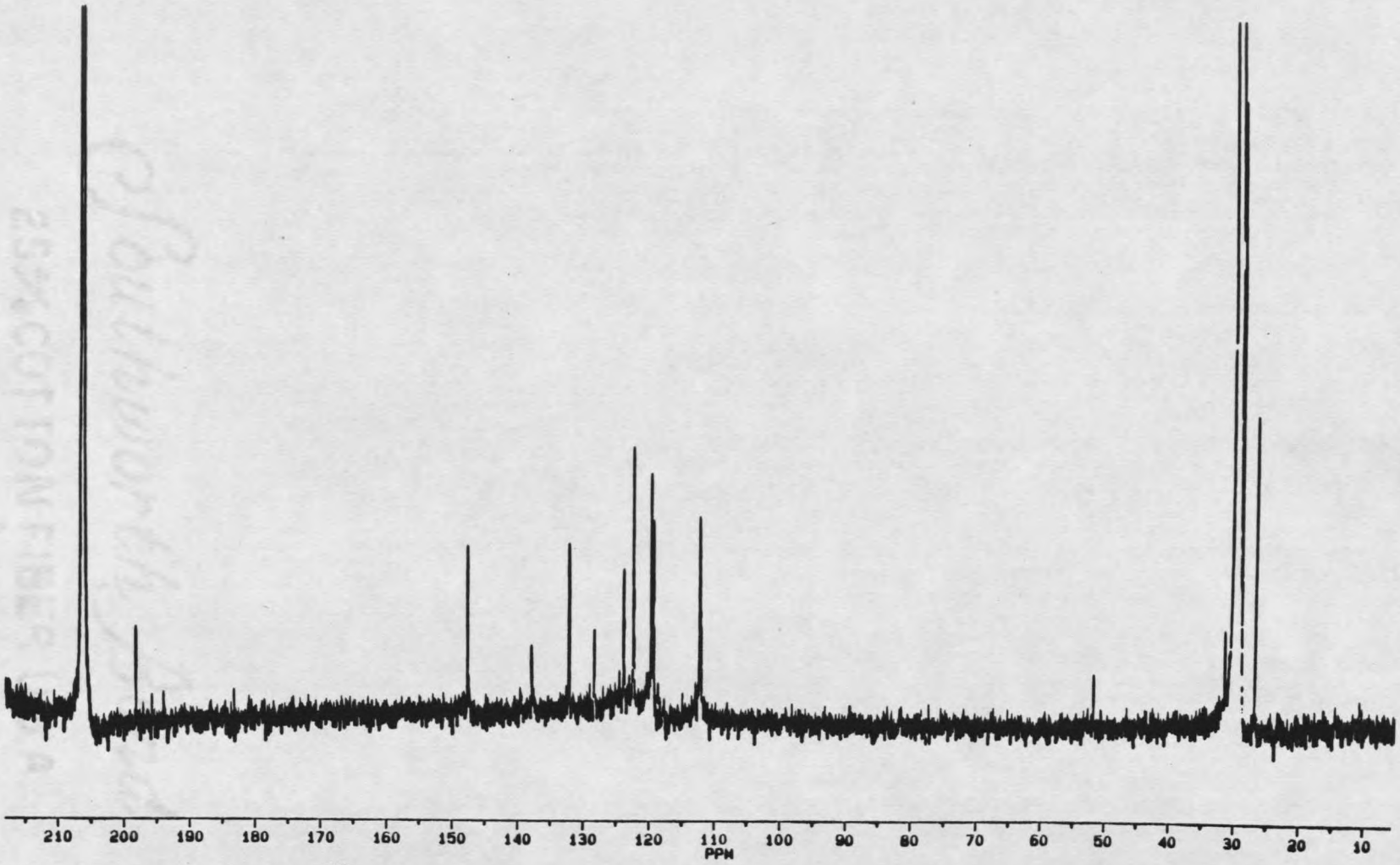


Figure 22. 125 MHz ^{13}C NMR spectrum of 21 in d_6-acetone.

δ198.1 in the ^{13}C NMR spectrum (Fig. 22) and a band at 1661 cm^{-1} in the IR spectrum for a α,β-unsaturated ketone.

The ^{1}H NMR spectrum (Fig. 21) contained spin-coupled aromatic protons at δ7.5(d), δ7.3(d) and δ6.9-7.1(m) indicative of an ortho disubstituted benzene ring. ^{13}C chemical shifts helped establish it as part of the indole structure (see Table 4).

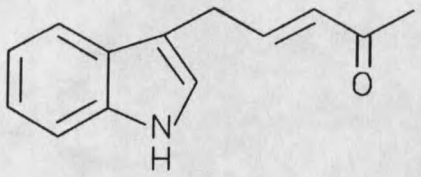
The proton at δ7.19 could again be at either C-2 or C-3. Its coupling constant of 2 Hz, however, coupled it to the amine proton, establishing its position at C-2.

The proton doublet at δ6.1 was coupled into the multiplet at δ6.9-7.1 by 16 Hz. The large J value of the two protons implied a trans double bond. Their chemical shift difference, however, was not as great as that seen for the previous two compounds, suggesting the double bond was not directly sandwiched between the carbonyl and the aromatic system. Chemical shift calculations (39) placed the alkene adjacent to the carbonyl and thus somewhat removed from the indole system. This arrangement was again supported by the α,β-unsaturated ketone seen in the IR spectrum at 1661 cm^{-1}.

Two signals in the ^{1}H NMR spectrum were still not assigned. This pair of doublets centered at δ3.7 had a coupling constant of 23 Hz, which implied geminal protons.

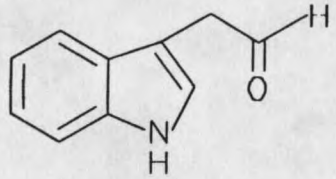
Each also had a coupling constant of 6 Hz, which coupled the methylene group into the multiplet at δ6.9-7.1 and hence the β proton of the α,β-unsaturated ketone. The ^1H chemical shift of the methylene group placed it between the indole and the α,β-unsaturated ketone.

Mass spectral fragmentation supported the side chain structure with first a loss of 43 for the methyl ketone, followed by a loss of 26 for the olefinic group. From the above data the compound was determined to be 5-(indol-3-yl)pent-3(E)-ene-2-one, **21**.



21

Once again **21** could possibly be an artefact of a condensation between indole 3-acetaldehyde, **22**, and acetone.



22

The fourth indole derivative was now easily identified. Mass spectral analysis (Fig. 23) gave an M+ peak at m/z 175 which required a molecular formula of $C_{10}H_9NO_2$ and seven sites of unsaturation. Fragmentation again was characteristic of an indole. ^1H NMR analysis (Fig. 24) showed four mutually coupled protons in the aromatic region at δ8.2(d), δ7.4(d) and δ7.15-7.25(m). This, along with the ^{13}C NMR spectrum (Fig. 25 and Table 4), helped support the indole structure. The proton at δ8.1 was again placed on the C-2 carbon.

With the indole accounting for C_8H_6N and six unsaturations, the C-3 substituent contained $C_2H_3O_2$ and one unsaturation. This implied either a methyl ester or an α-hydroxyketone. Indeed, mass spectral fragmentation supported this with a loss of M^+-31 for $\cdot OCH_3$ followed by a loss of 28 for CO. The ^1H NMR spectrum showed a singlet at δ4.67, which was too far downfield for the methyl of a methyl ester but seemed correct for a methylene influenced by both a ketone and a hydroxyl group. The peak at δ66.3 in the ^{13}C NMR spectrum was also indicative of a methylene attached to a heteroatom. The data supported the structure as 3-(hydroxyacetyl)indole, 23.

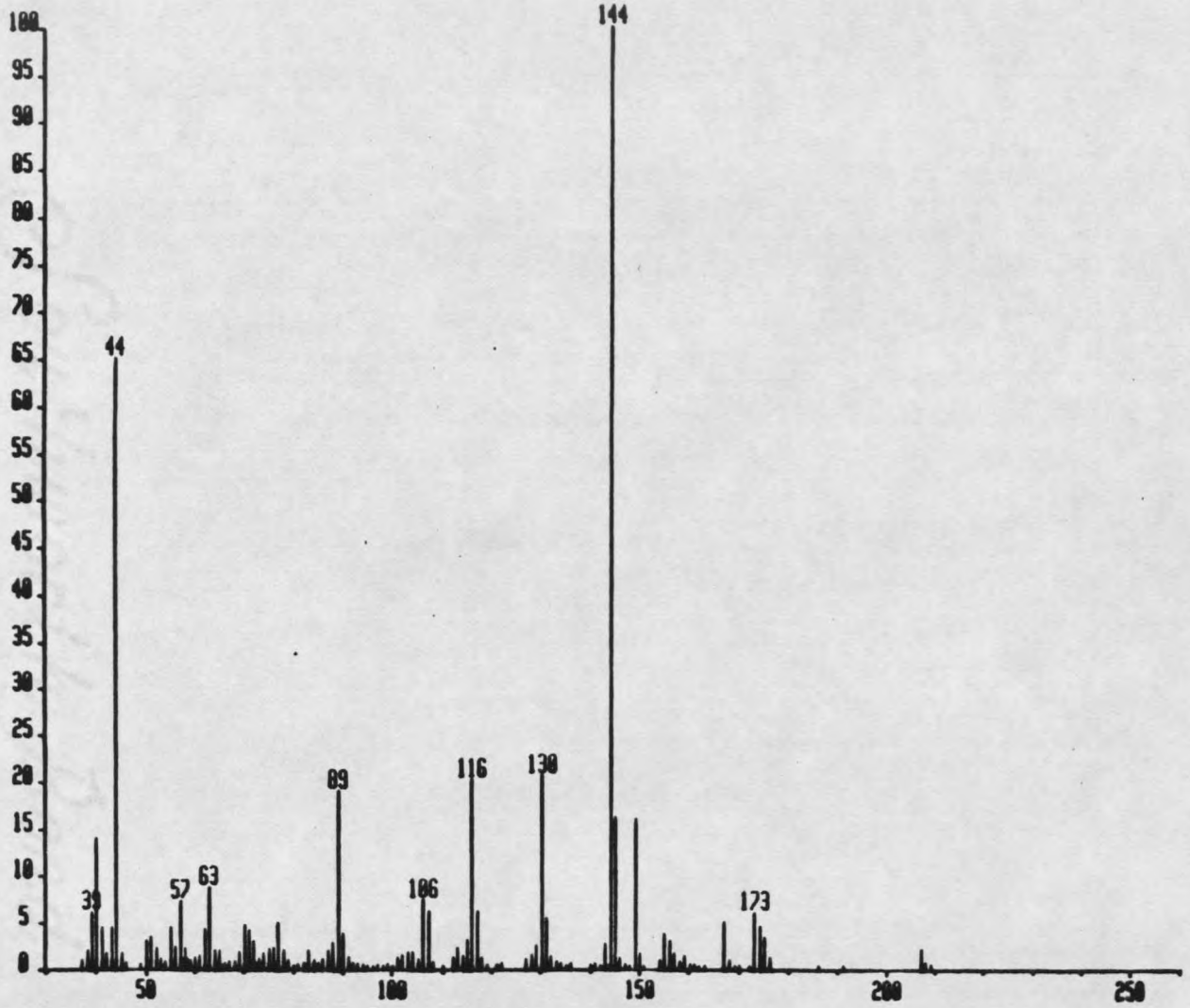


Figure 23. EI mass spectrum of 23.

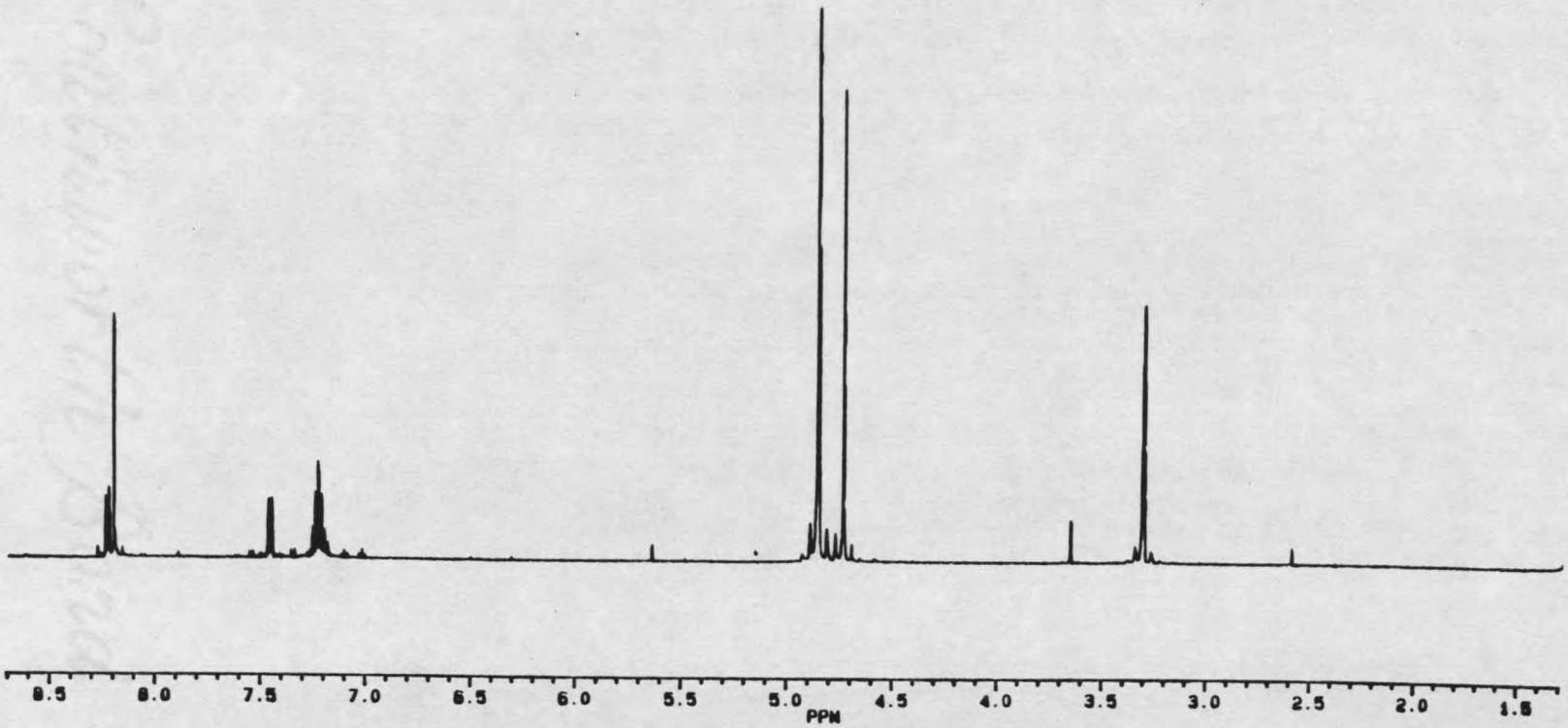


Figure 24. 300 MHz ^{1}H NMR spectrum of 23 in CD_3OD.

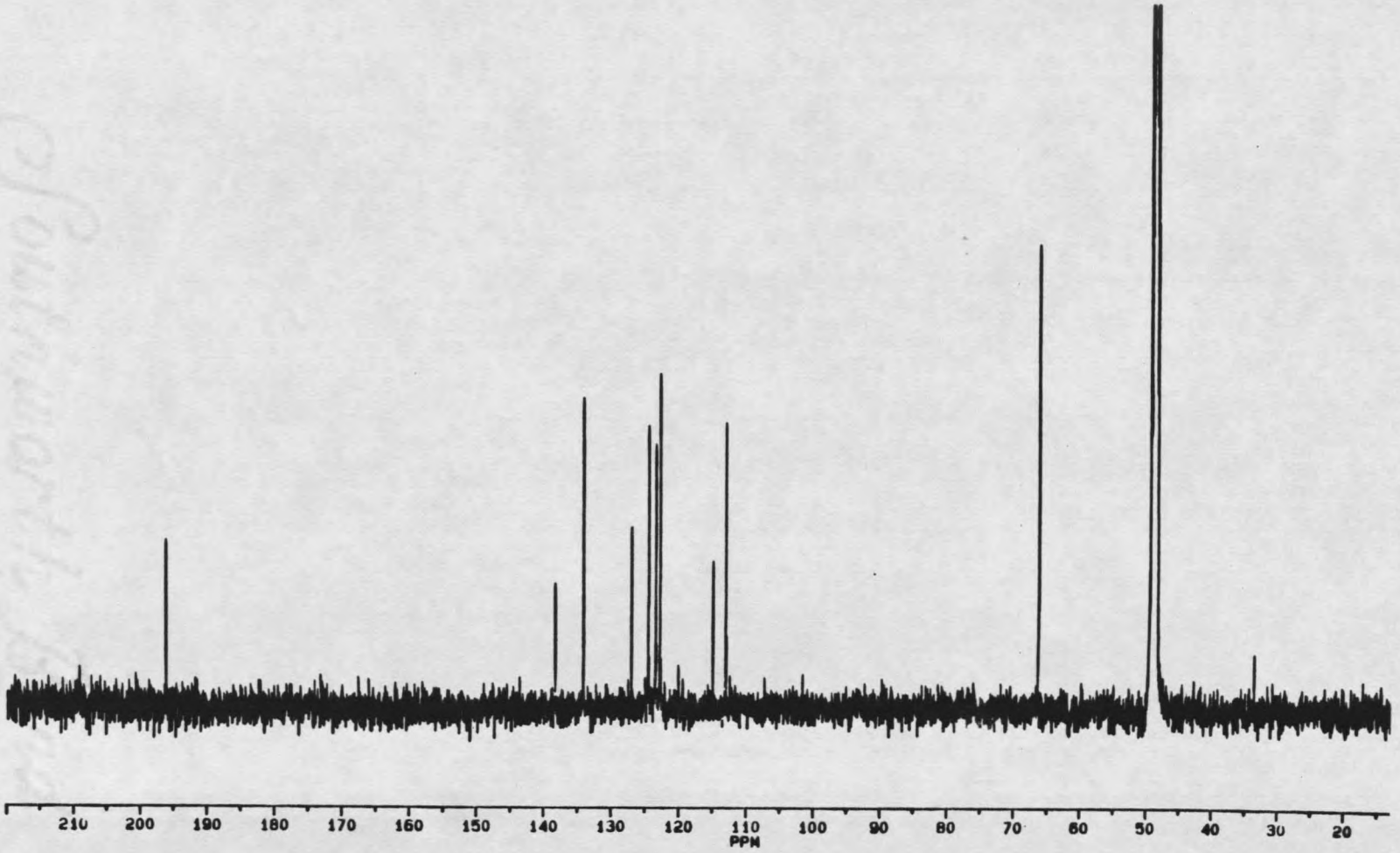
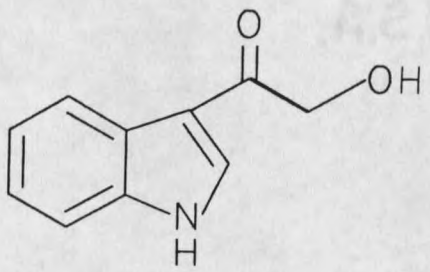


Figure 25. 125 MHz ^{13}C NMR spectrum of 23 in CD_3OD.



23

Two of the aromatics from ***T. ignis*** were a carbazole and a β-carboline. The first of these to be characterized was **24**. Mass spectral analysis (Fig. 26) revealed a molecular ion at m/z 181, which required a molecular formula of $C_{13}H_{11}N$ and nine double bond equivalents. Fragmentation was limited and uninformative.

The ^{1}H NMR spectrum (Fig. 27) showed two sets of spin coupled aromatic protons. A $^{1}H,^{1}H$ COSY experiment (Fig. 28) sorted out the couplings and revealed the presence of one ortho disubstituted and one 1,2,3-trisubstituted aromatic ring. This accounted for all but one carbon, which appeared as a methyl at δ2.56, leaving only the nitrogen and one unsaturation to complete the structure. Another ring had to be present.

A ^{13}C NMR analysis (Fig. 29) suggested an indole and

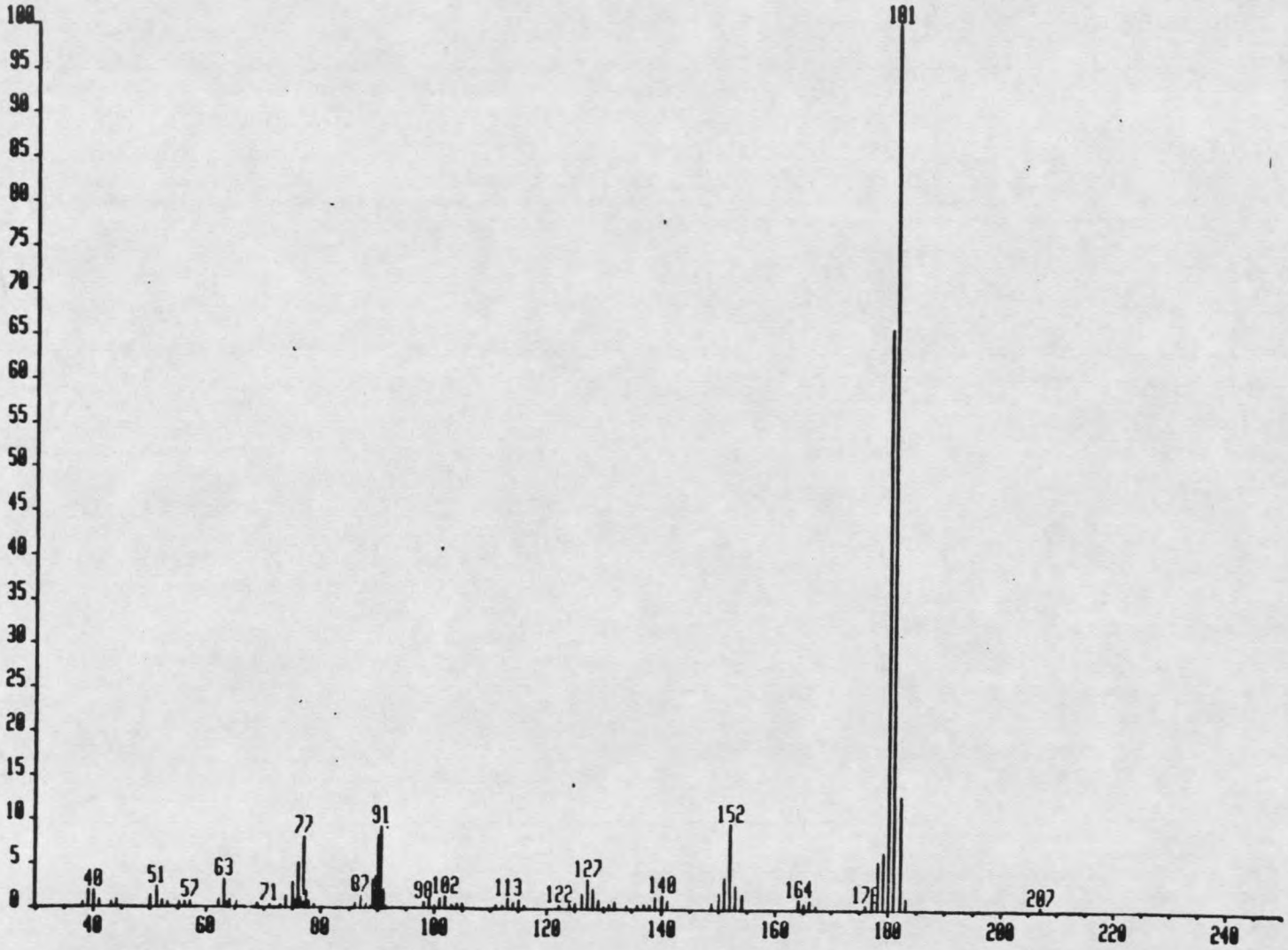


Figure 26. EI mass spectrum of 24.

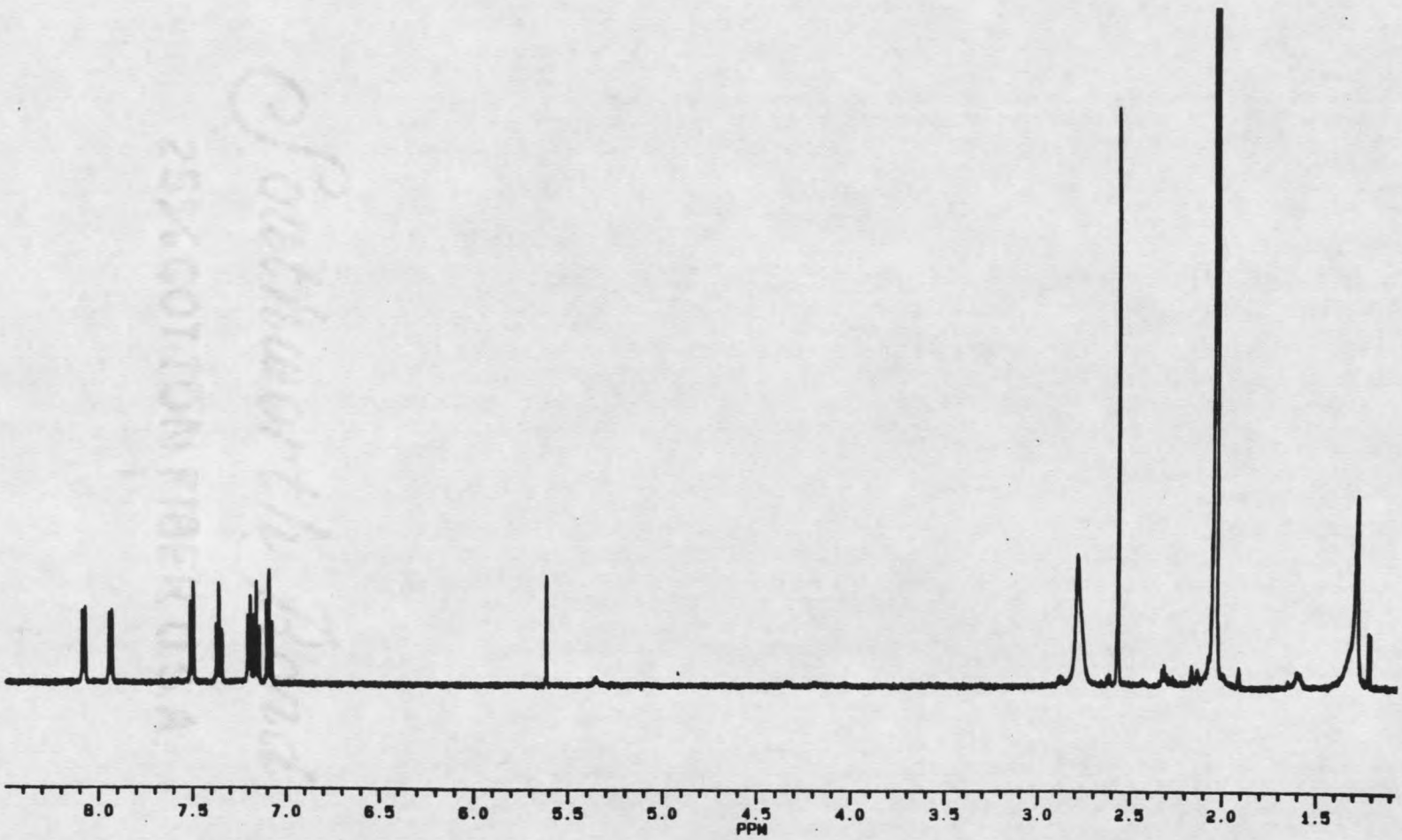


Figure 27. 500 MHz ^{1}H NMR spectrum of **24** in d_6-acetone.

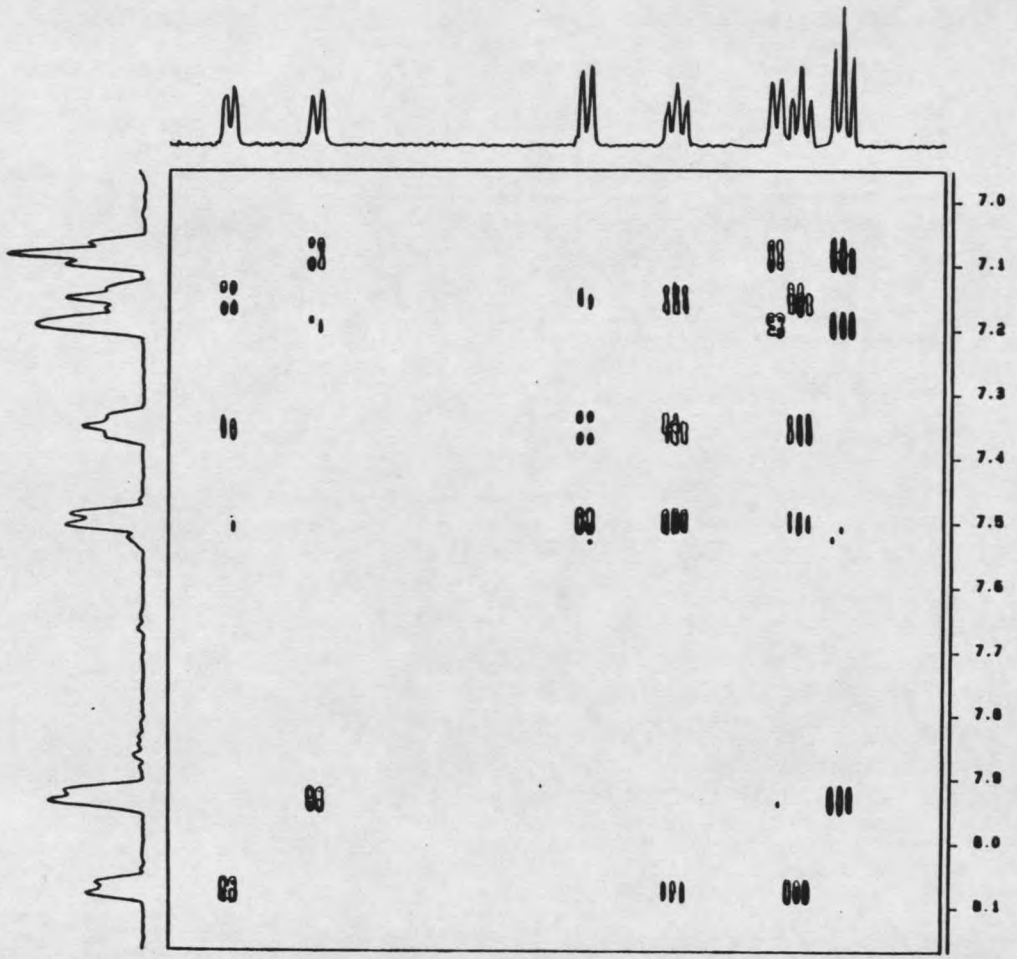


Figure 28. 500 MHz COSY spectrum of **24** in d_6-acetone.

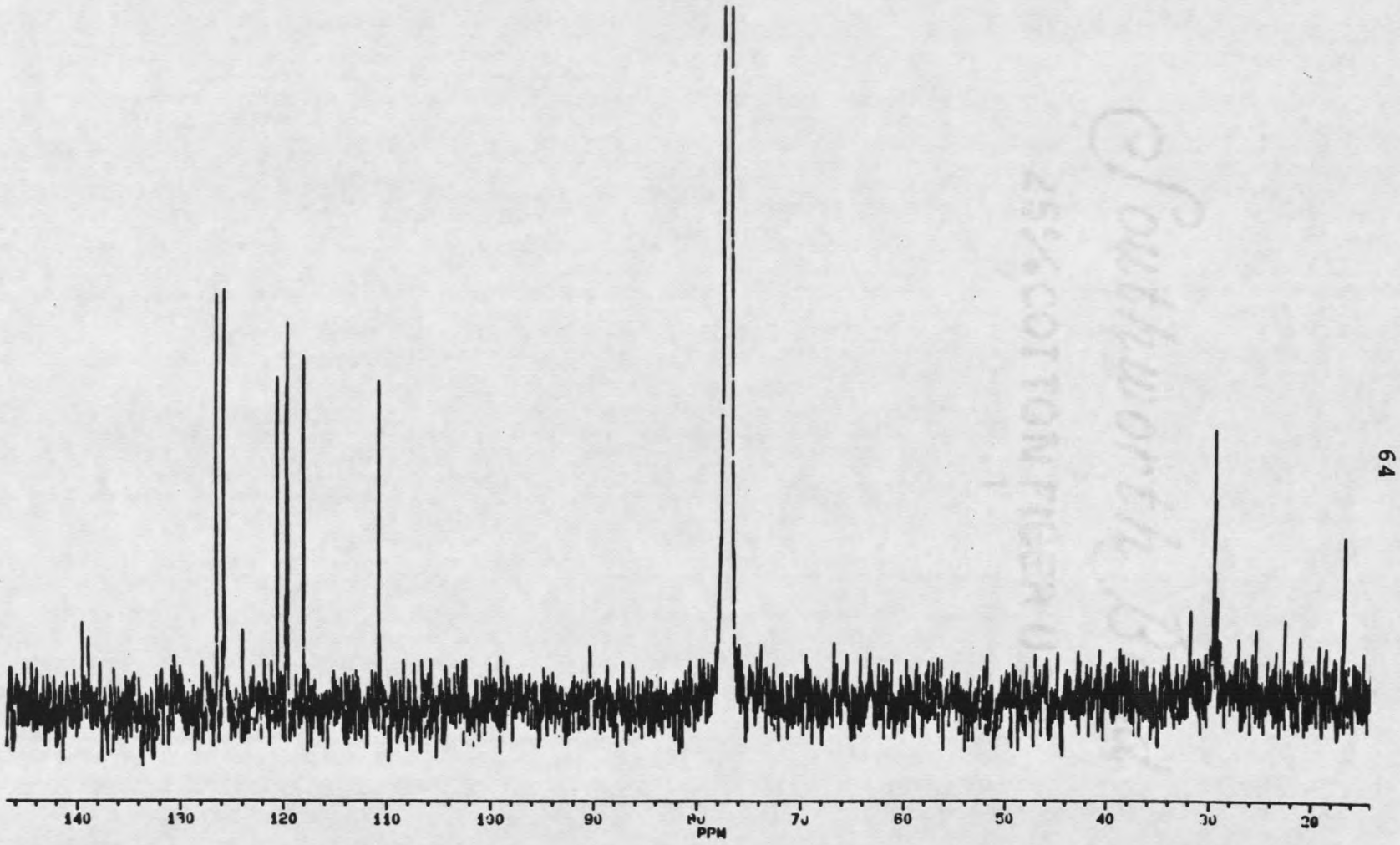
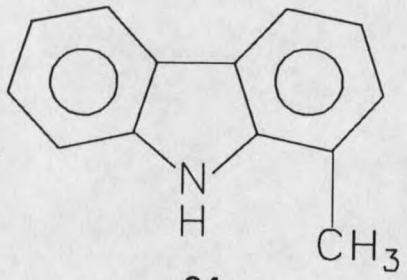


Figure 29. 125 MHz ^{13}C NMR spectrum of **24** in $CDCl_3$.

with what was known from the ^{1}H NMR spectrum, a carbazole was suspected (Table 5). Indeed, comparison of data matched that of the known 1-methyl carbazole (44, 45).

As further support to the structure, a UV spectrum of carbazole was run and compared to that of the natural product. Since it was known the methyl group would not have a great effect on the UV (46), the spectra should be in close agreement, as indeed they were. The structure was thus shown to be 1-methylcarbazole, **24**.



24

The mass spectrum of the second alkaloid, **25** (Fig. 30), showed an M^{+} peak at m/z 224 which required a molecular formula of $C_{13}H_{10}N_2O$ and 10 double bond equivalents. The IR spectrum revealed two significant peaks at 3444 and 1671 cm^{-1}, denoting the presence of an indole amine and an α,β-unsaturated ketone, respectively. A peak at δ203.3 in the ^{13}C NMR spectrum (Fig. 31) confirmed that a ketone was present.

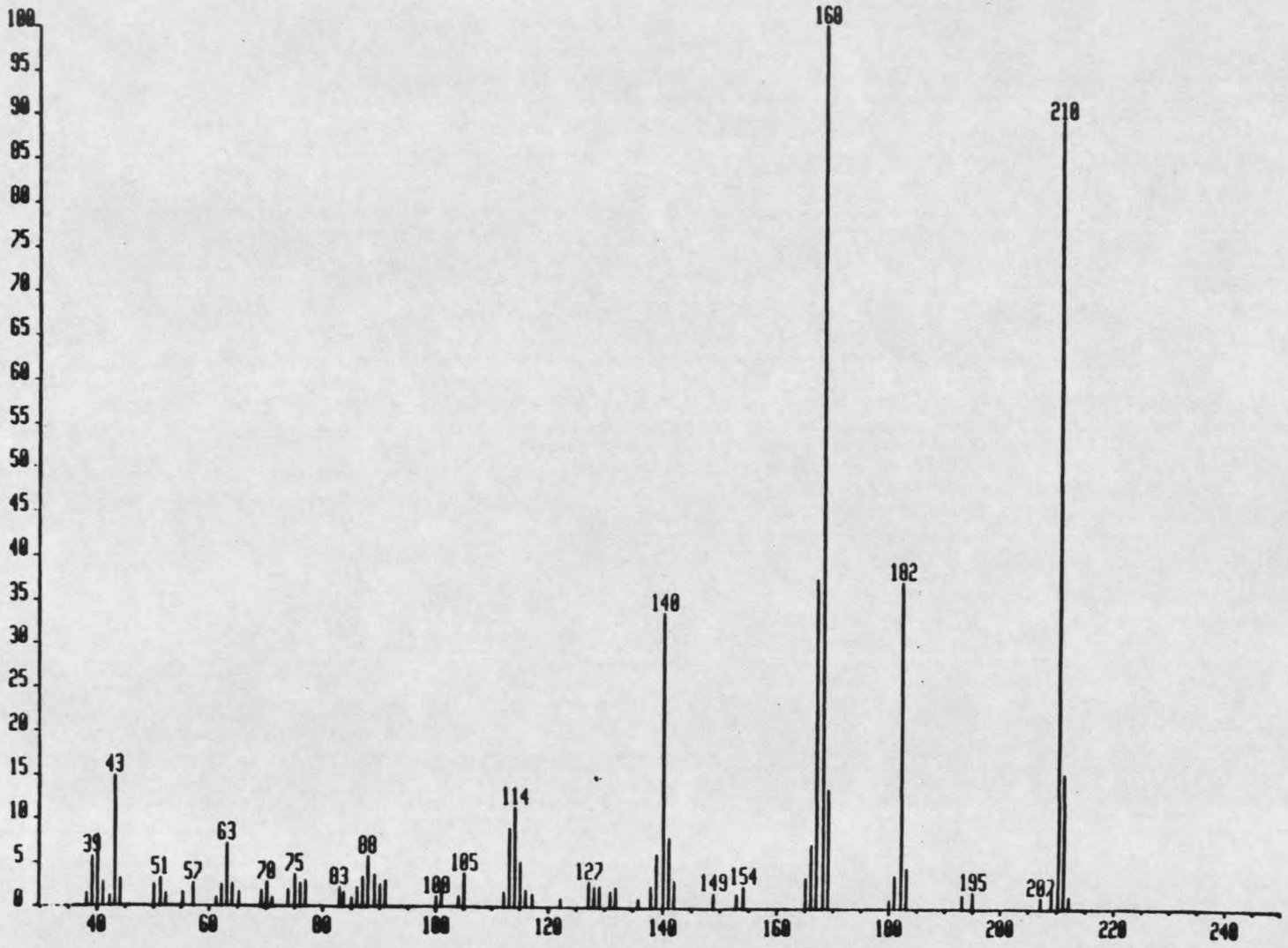


Figure 30. EI mass spectrum of 25.

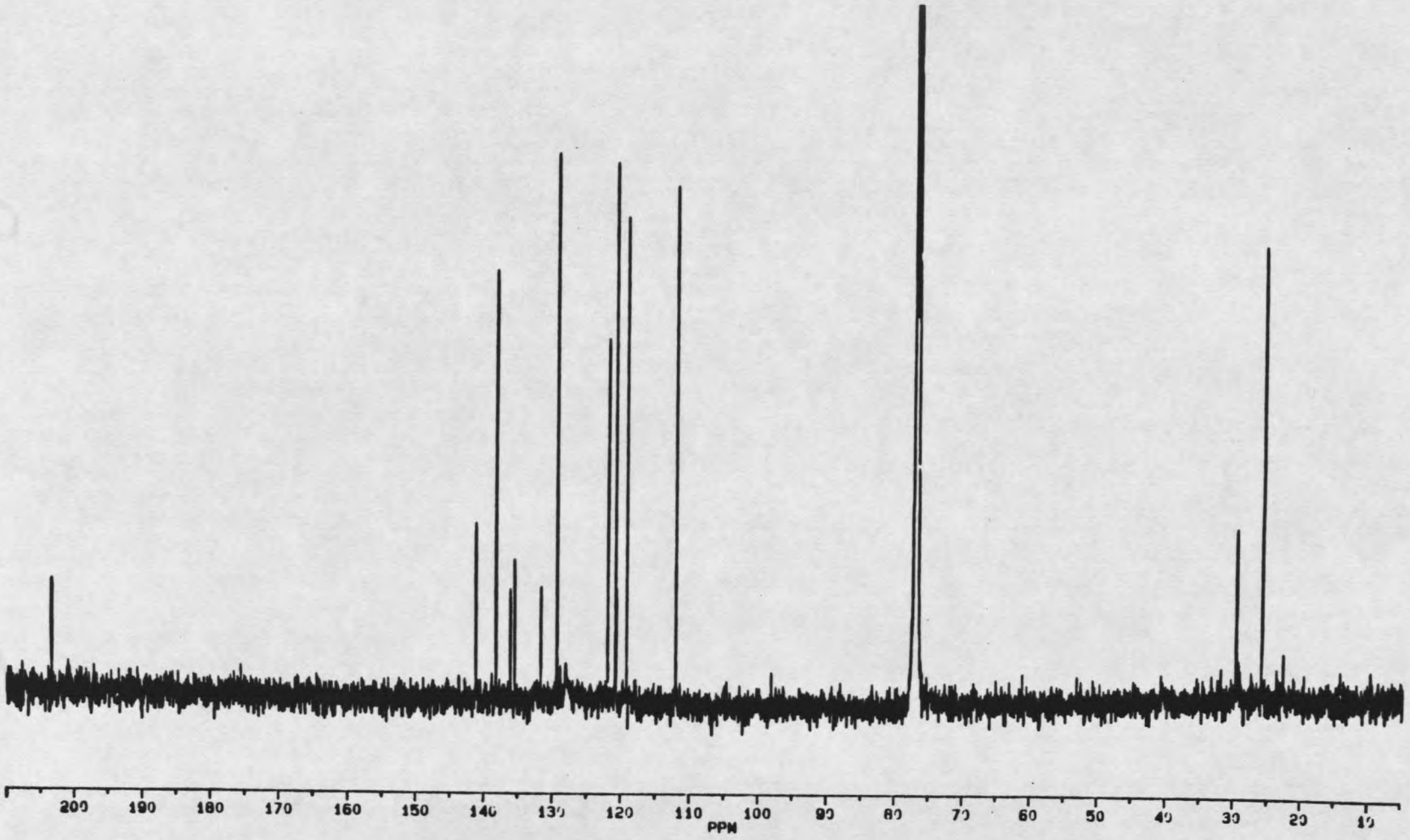


Figure 31. 125 MHz ^{13}C NMR spectrum of 25 in $CDCl_3$.

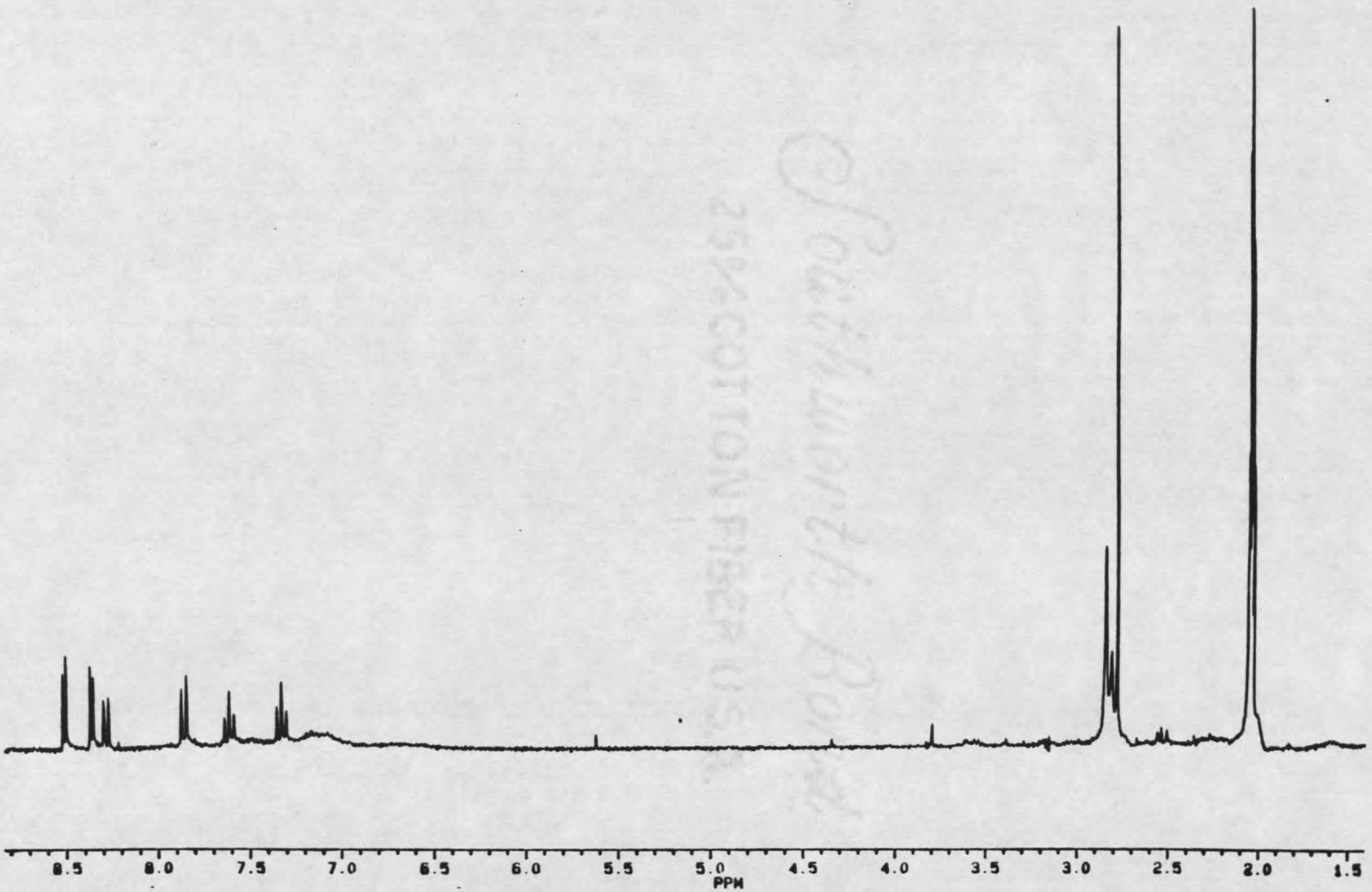
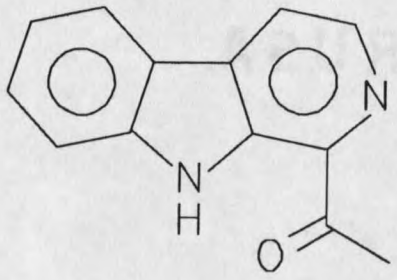


Figure 32. 500 MHz ^{1}H NMR spectrum of 25 in d_6-acetone.

The ^{1}H NMR spectrum (Fig. 32) and $^{1}H,^{1}H$ COSY experiment again showed two sets of mutually coupled aromatic protons. The first set at δ8.3, δ7.8, δ7.6 and δ7.3 belonged to an ortho disubstituted ring. The ^{1}H and ^{13}C chemical shifts (Table 5) indicated an indole. The chemical shifts of the coupled protons at δ8.5 and δ8.4 placed them under the direct influence of nitrogen. At this point a β-carboline structure was suspected and NMR data were found to match closely those of known 1-substituted β-carbolines (47). A methyl ketone at C-1 would complete the structure and, in fact, matched the data of the isolated product. Thus, 25 was 1-acetyl-β-carboline.



25

Table 5. NMR Assignments for 24 and 25[a,b].

Carbon #	24		25	
	^{13}C	^{1}H	^{13}C	^{1}H
1	c	- - -	136.1	- - -
2	119.6	7.20 d	- - -	- - -
3	119.5	7.08 t	138.2	8.50 d
4	126.4	7.90 d	120.7	8.40 d
4a	119.7	- - -	131.5	- - -
4b	123.8	- - -	120.6	- - -
5	125.6	8.08 d	121.8	8.30 d
6	117.9	7.16 t	119.1	7.30 t
7	120.5	7.35 t	129.3	7.60 t
8	110.7	7.50 d	112.0	7.80 d
8a	138.9	- - -	141.1	- - -
9a	139.4	- - -	135.4	- - -
Me	16.8	2.56 s	25.9	2.80 s
CO			203.3	- - -

[a]recorded in d_6-acetone
[b]assignments and numbering of carbons based on known chemical shifts of carbazole and β-carboline and may be interchangeable
[c]carbon missing from ^{13}C NMR spectrum

Several simple benzenoid aromatics were isolated from ***T. ignis***. The first, **26**, gave a molecular ion at m/z 135 (Fig. 33), which required a molecular formula of C_8H_9NO and five double bond equivalents. The IR spectrum showed two sharp, intense peaks at 3519 and 3412 cm^{-1}, which were characteristic of a primary amide. Also in the IR spectrum were two peaks at 1687 and 1586 cm^{-1}, again representative of a primary amide.

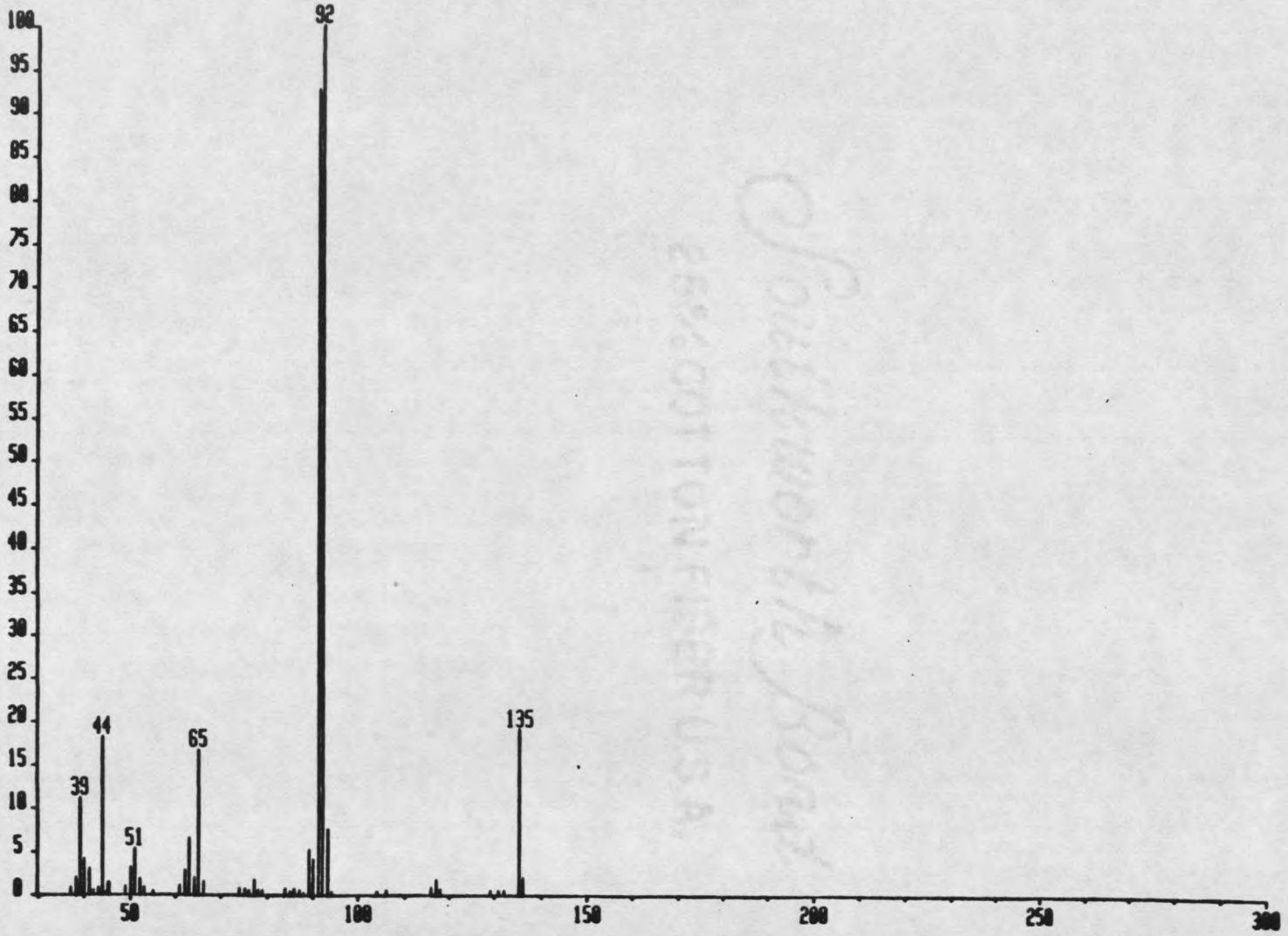
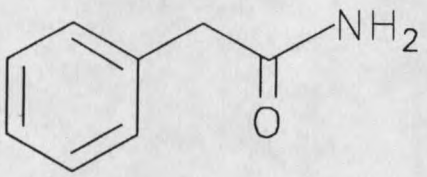


Figure 33. EI mass spectrum of 26.

The ^{1}H NMR spectrum (Fig. 34) was not complicated. The 5H multiplet at δ7.3-7.4 indicated a monosubstituted benzene ring and the broad singlets at δ5.85 and δ5.5 supported the IR data for a primary amine. These two part structures left only a $-CH_2-$ to be assigned. The chemical shift of the singlet at δ3.0 placed the methylene between the aromatic ring and the amide. ^{13}C NMR spectrum (Fig. 35) and a MS fragment ion at m/z 91, which is characteristic of $C_7H_7^+$, supported the structure. Compound **26** was, then, 2-phenylacetamide.



26

Compound **27** appeared to be very similar to **26**, as seen by its ^{1}H NMR (Fig. 36) and ^{13}C NMR (Fig. 37) spectra. A mass spectrum (Fig. 38) gave a molecular ion at m/z 136, which was 1 amu more than **26**. Fragmentation showed a loss of 45, which could represent a COOH group. The m/z 91 fragment was again characteristic of C_7H_7+, indicating

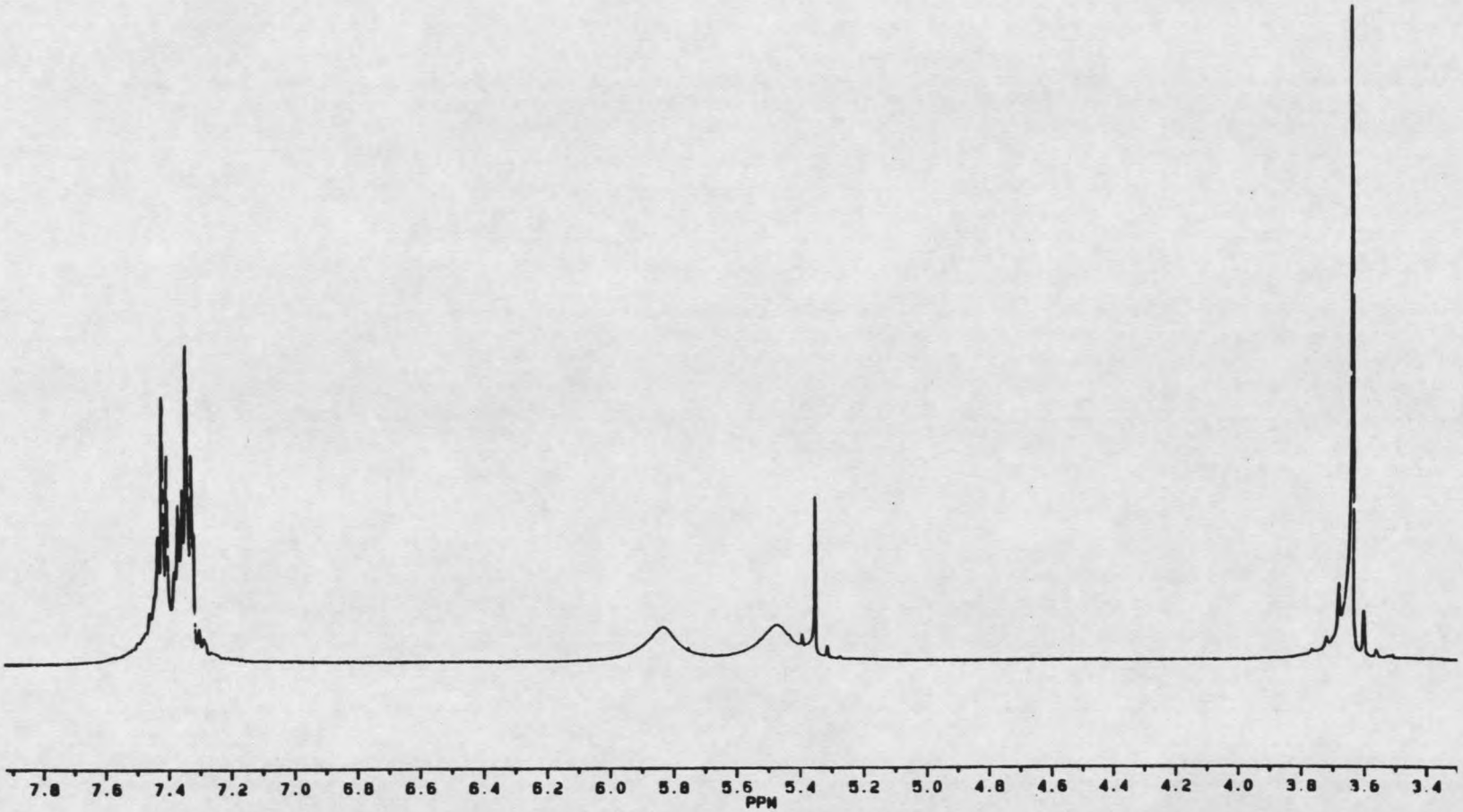


Figure 34. 300 MHz ^{1}H NMR spectrum of **26** in d_6-acetone.

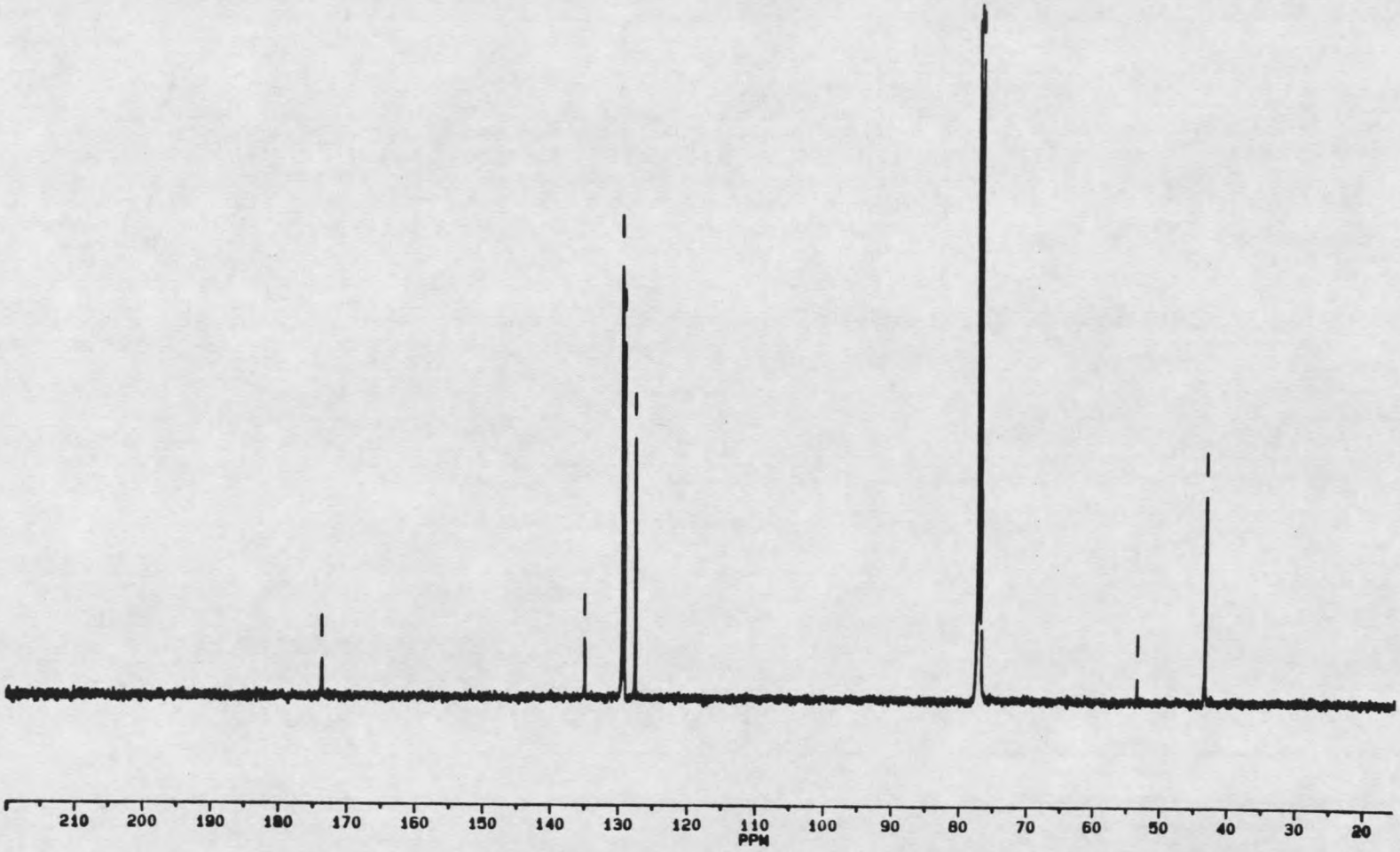


Figure 35. 125 MHz ^{13}C NMR spectrum of **26** in $CDCl_3$.

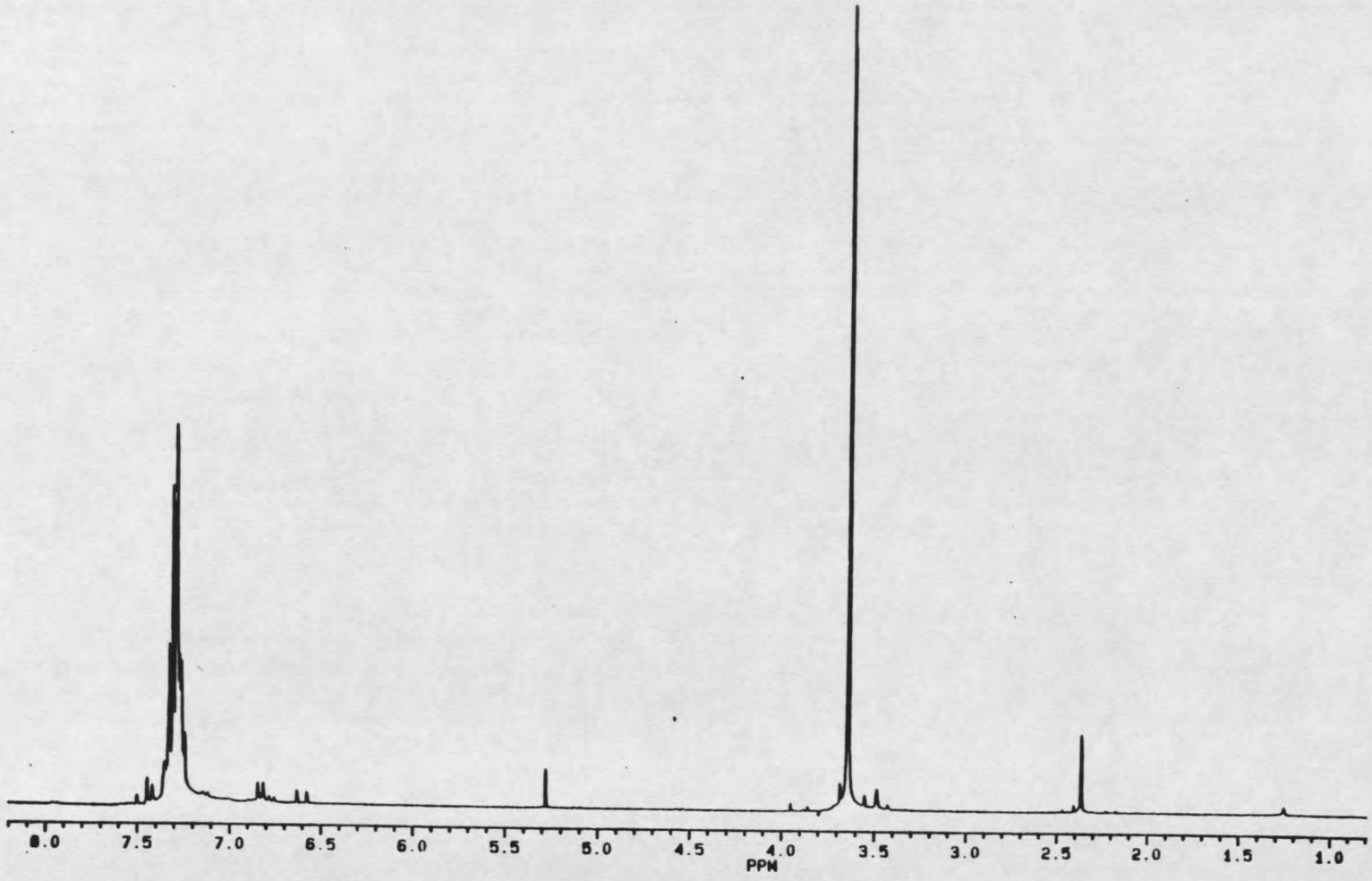


Fig. 36. 300 MHz ^{1}H NMR spectrum of 27 in $CDCl_3$.

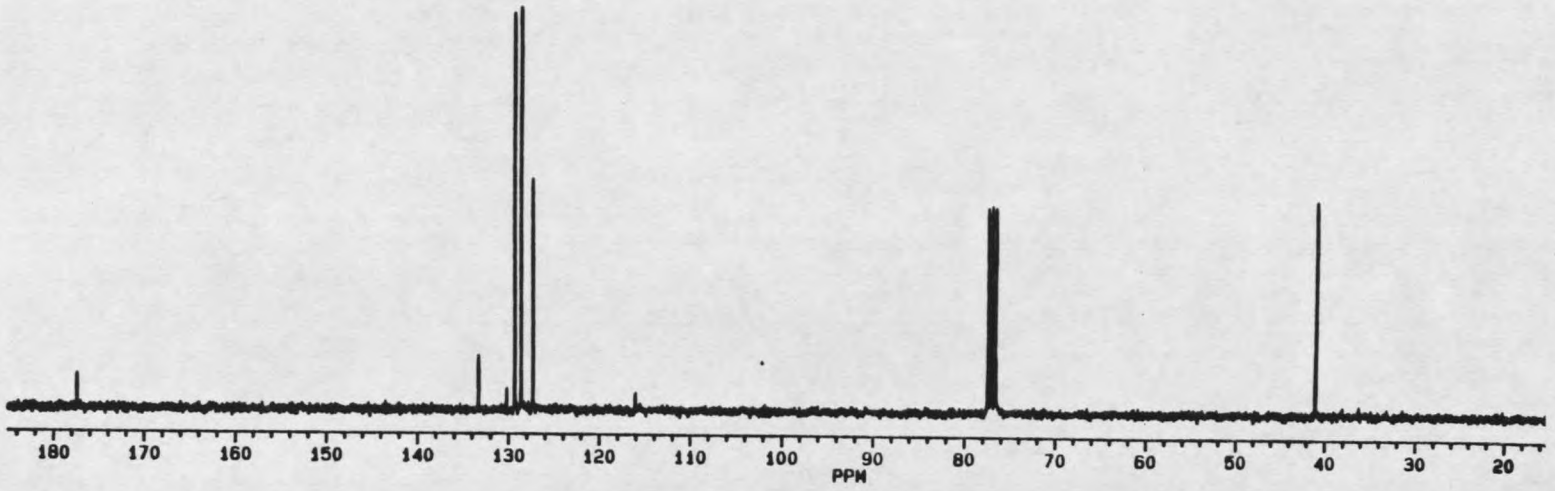


Fig. 37. 125 MHz ^{13}C NMR spectrum of 27 in $CDCl_3$.

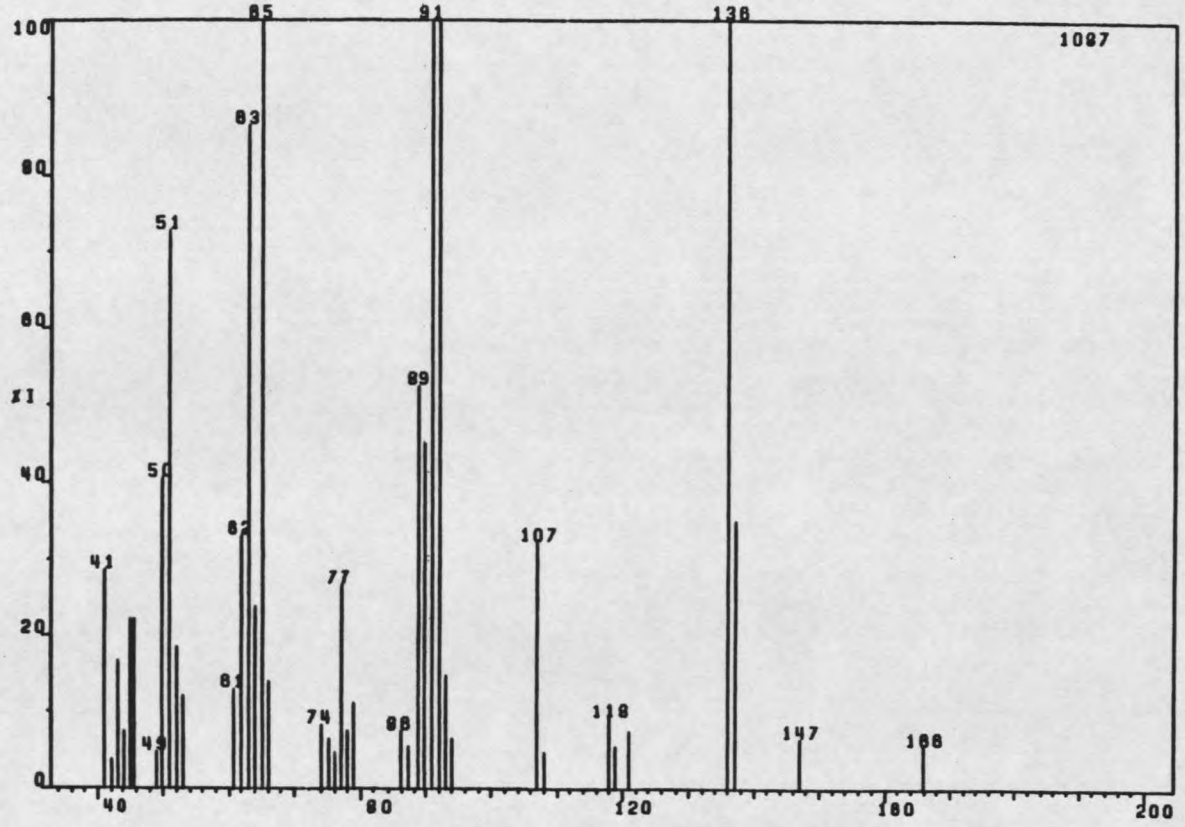
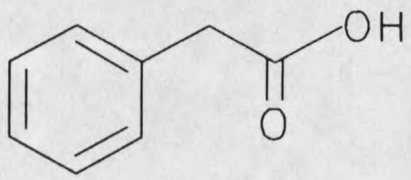


Fig. 38. EI mass spectrum of 27.

$C_6H_5CH_2X$ was present. A look at the IR spectrum revealed a broad band whose ν_{max} at 3010 cm^{-1}, along with a band at 1710 cm^{-1}, supported the presence of a carboxylic acid. High resolution mass spectral data confirmed the structure of **27**, with a molecular formula of $C_8H_8O_2$, to be 2-phenylacetic acid. ^1H and ^{13}C NMR data matched those of the known compound (48).



27

Compound **28** was somewhat more complicated. Mass spectral analysis (Fig. 39) gave a molecular ion at m/z 188, which required a molecular formula of $C_{13}H_{16}O$ and 6 double bond equivalents. The IR spectrum showed only one band of significance, at 1673 cm^{-1}, suggesting an α,β-unsaturated ketone.

The ^1H NMR spectrum (Fig. 40) showed four downfield doublets. Those at δ7.37 and δ7.04 were coupled by 8 Hz. Their coupling constant and chemical shifts, along with the chemical shifts seen in the ^{13}C NMR spectrum (Fig. 41)

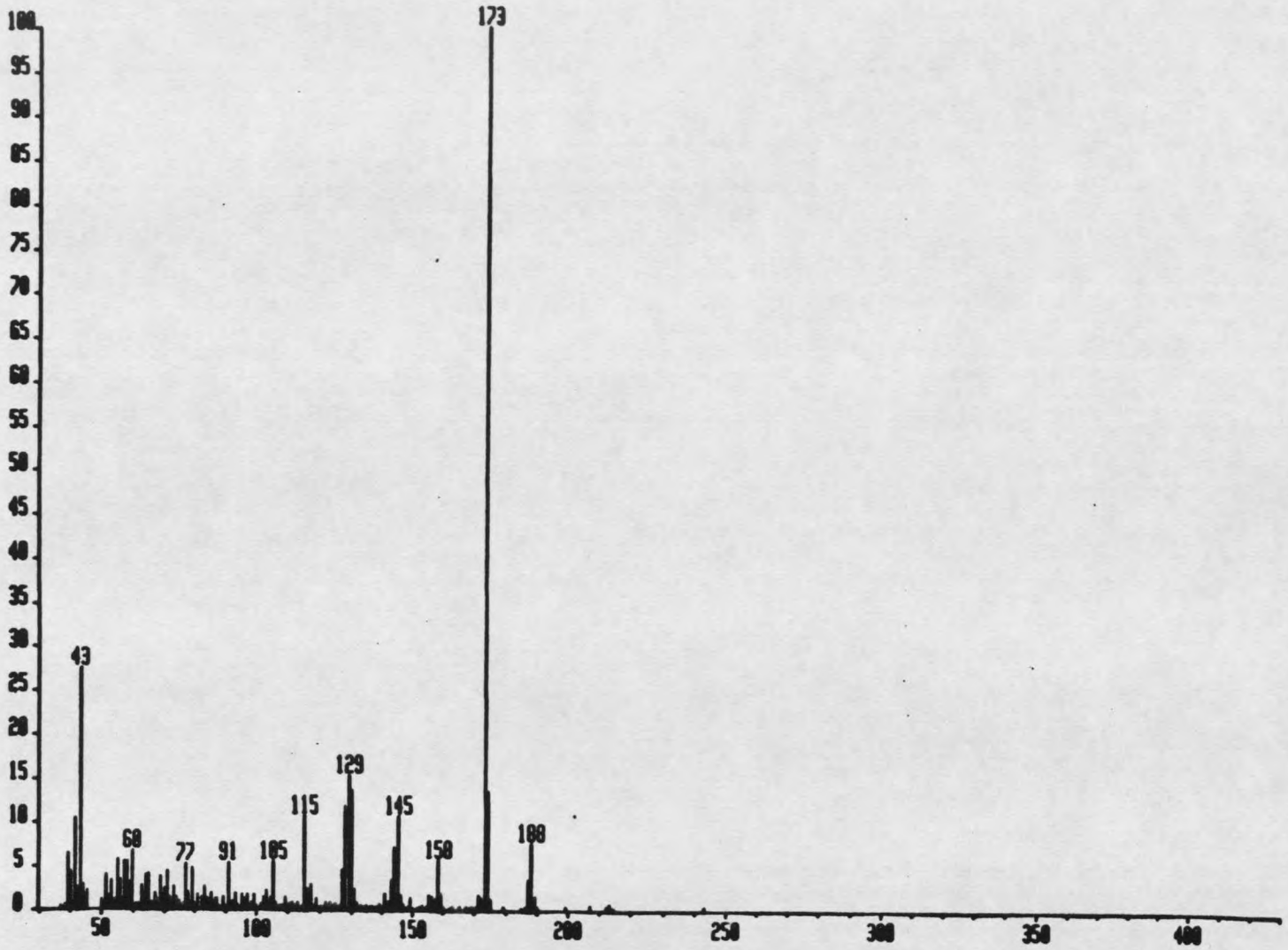


Figure 39. EI mass spectrum of 28.

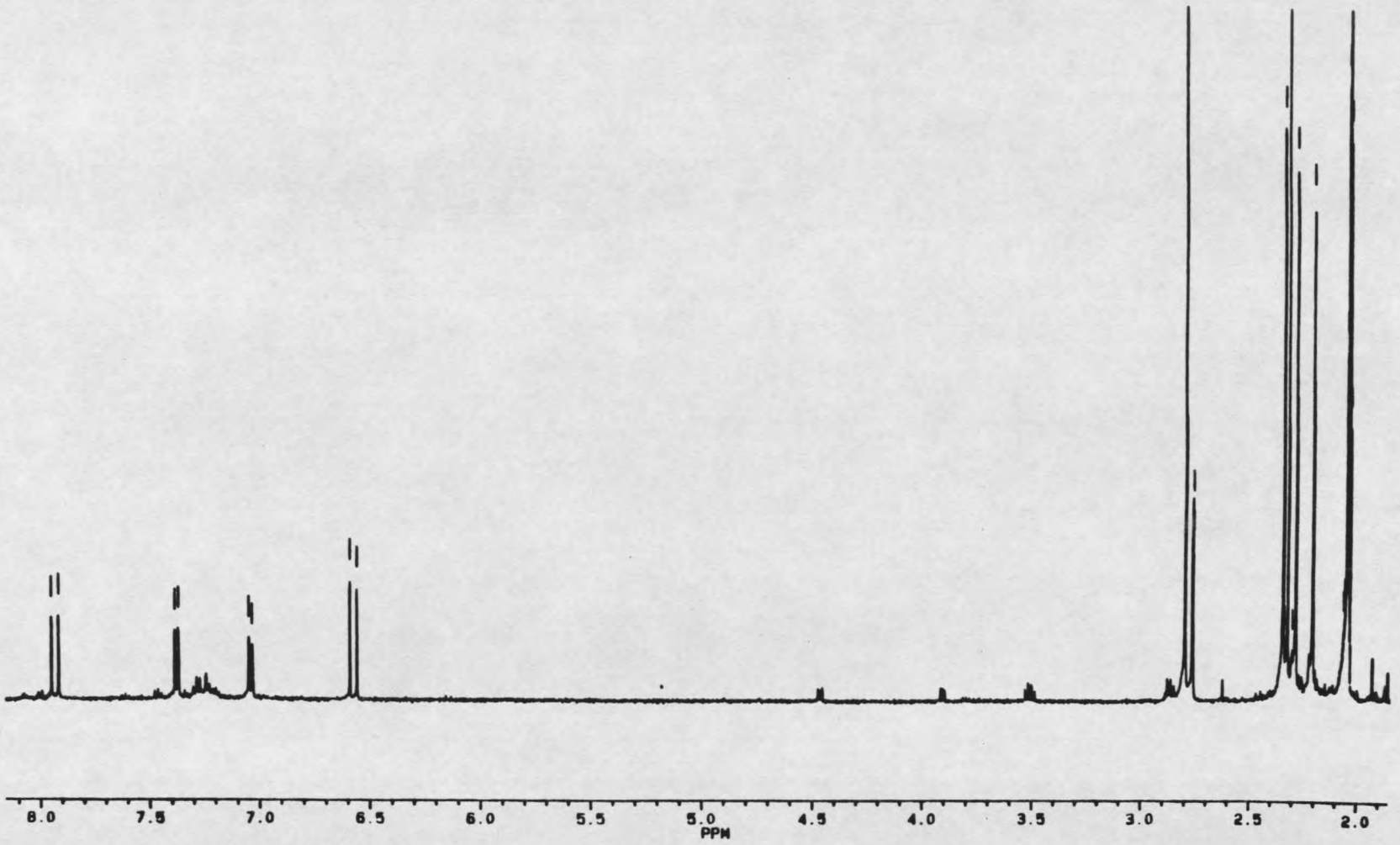


Figure 40. 500 MHz ^{1}H NMR spectrum of **28** in d_6-acetone.

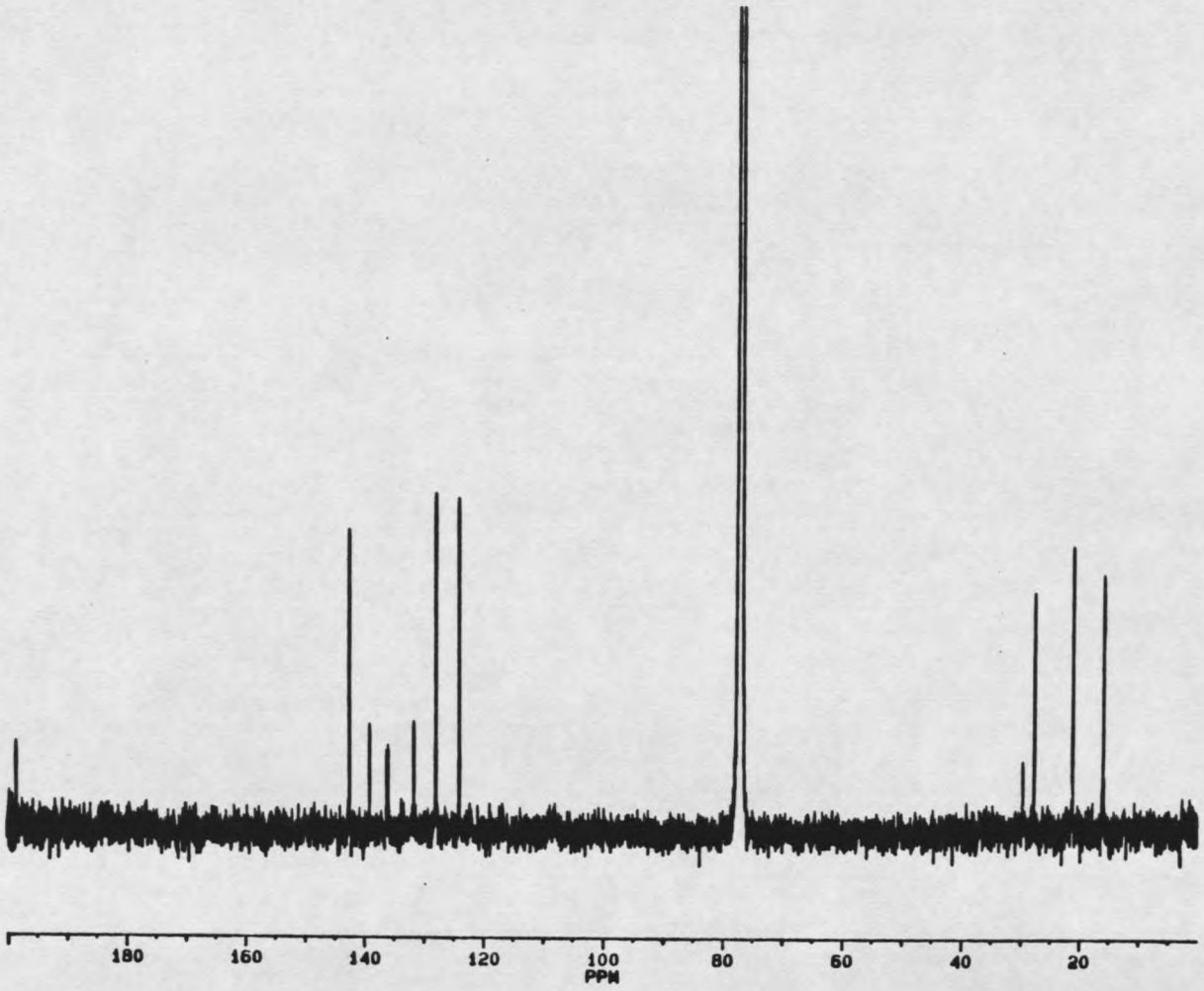


Figure 41. 125 MHz ^{13}C NMR spectrum of **28** in $CDCl_3$.

implied that they were ortho protons on a benzene ring. The other proton doublets, at δ7.93 and δ6.57, had a coupling constant of 16 Hz. This indicated a trans double bond and hence these protons had to be part of a substituent. Since no other protons were in the aromatic region, the benzene ring had to be a 1, 2, 3, 4, tetrasubstituted system. The only other signals in the ^{1}H NMR spectrum were four 3H singlets at δ2.34, δ2.32, δ2.28 and δ2.20.

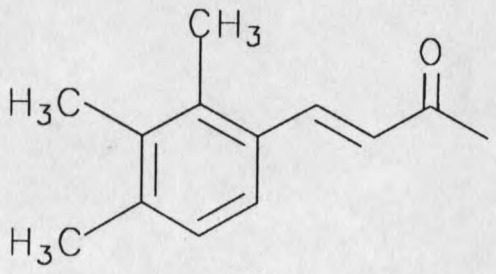
The difference in chemical shifts of the protons on the trans olefin indicated that the bond was polarized. The shifts, as seen in previous compounds, placed the double bond between the aromatic ring and a carbonyl. The IR spectrum had already supported an α,β-unsaturated ketone structure and a peak at δ198.4 in the ^{13}C NMR spectrum confirmed that a ketone was present.

One of the methyl groups would complete the side chain (as a methyl ketone) and the other methyls would fulfill the substitution pattern of the ring. The problem remained in placing the unsaturated methyl ketone and three methyl groups on the ring in the correct pattern. The protons were known to be ortho so two possibilities were evident. The methyls could be placed at C-1, 2 and 3 with the unsaturated ketone at C-4 or the methyls could be

positioned at C-1, 2 and 4 with the olefinic ketone at C-3.

The chemical shifts of the ring protons helped establish the positioning of the groups. The proton at δ7.37 was deshielded and hence should have an electron withdrawing group, the unsaturated ketone, ortho to it. On the other hand, the proton at δ7.04 was somewhat shielded and should have an electron donating group, a methyl, ortho to it.

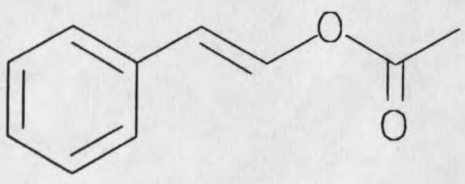
To help confirm these positionings, nOe experiments were run. A 6.3% nOe between the α-olefinic proton at δ6.57 and the ring proton at δ7.37 firmly established the ortho positioning of this ring proton to the side chain. A 6.4% nOe between the β-olefinic proton at δ7.93 and the methyl at δ2.34 placed this methyl ortho to the side chain. The methyl at δ2.28 was established as ortho to the ring proton at δ7.04 by an nOe of 5.4% between them. This data indicated the structure to be 4-(2′,3′,4′-trimethylphenyl)-but-3(E)-en-2-one, **28**.



28

The mass spectrum (Fig. 42) of the fourth benzenoid compound showed a molecular ion at m/z 162. High resolution mass spectral data gave a molecular formula of $C_{10}H_{10}O_2$, which required 6 sites of unsaturation. Fragmentation showed a loss of 15, followed by a loss of 44, indicating a methyl ester. The IR spectrum supported the presence of an ester with a band at 1760 cm^{-1}, with a vinyl ester being most probable. (Although the carbonyl carbon is missing from the ^{13}C NMR spectrum, IR and MS data firmly establish the presence of the ester.)

A ^{1}H NMR analysis (Fig. 43) supported this with two olefinic protons at δ7.60 and δ6.77. A coupling constant of 16 Hz indicated the olefin was trans. The methyl singlet appeared at δ2.33. The two downfield multiplets at δ7.68 and δ7.43, along with the ^{13}C NMR spectrum (Fig. 44), indicated an aromatic ring was present. Together the data showed **29** to be 2-phenylethenyl acetate.



29

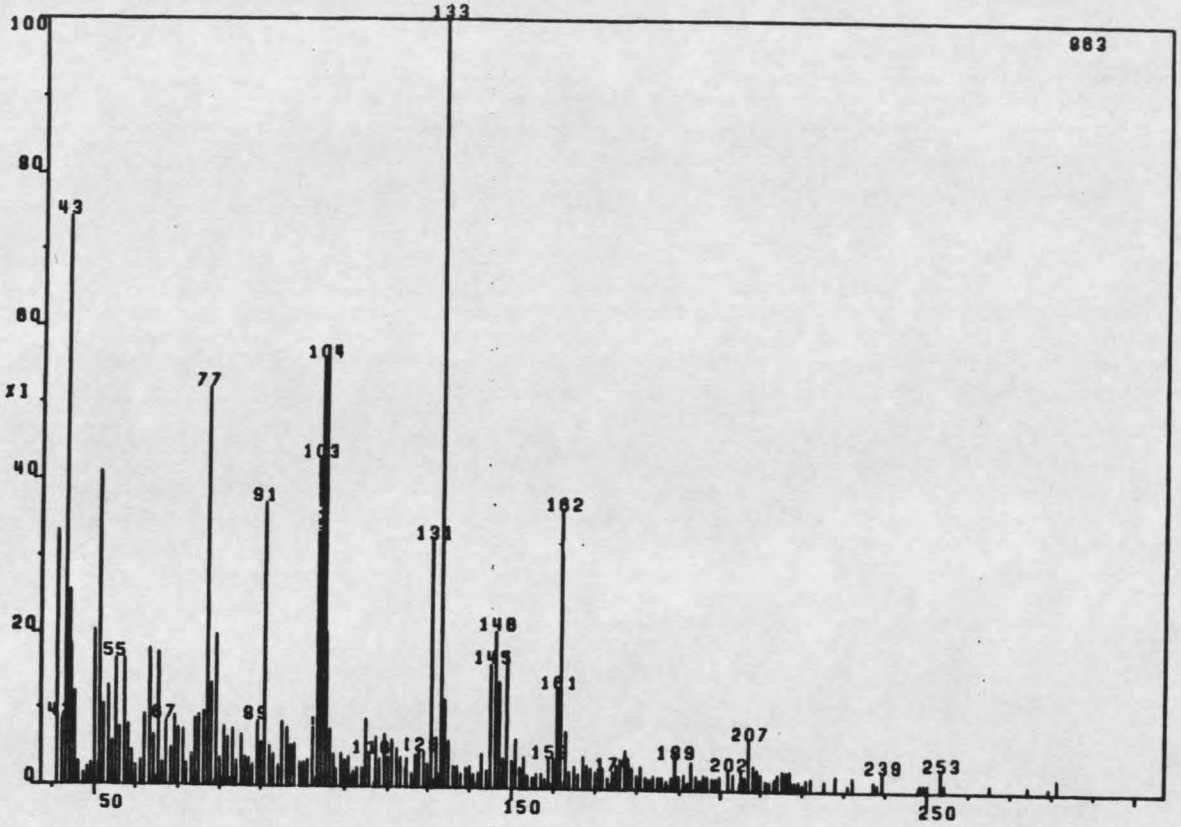


Fig. 42. EI mass spectrum of 29.

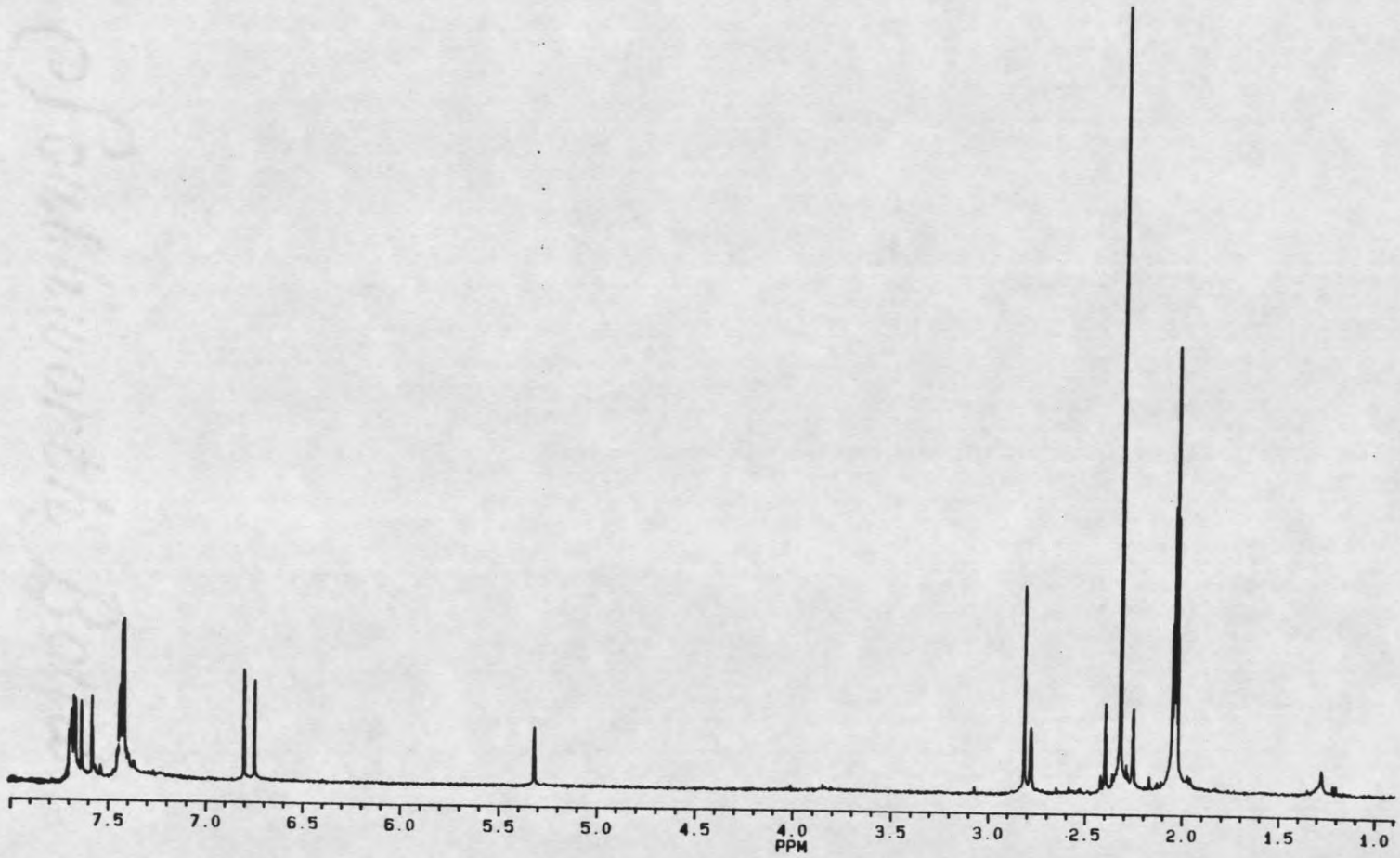


Fig. 43. 300 MHz ^{1}H NMR spectrum of **29** in d_6-acetone.

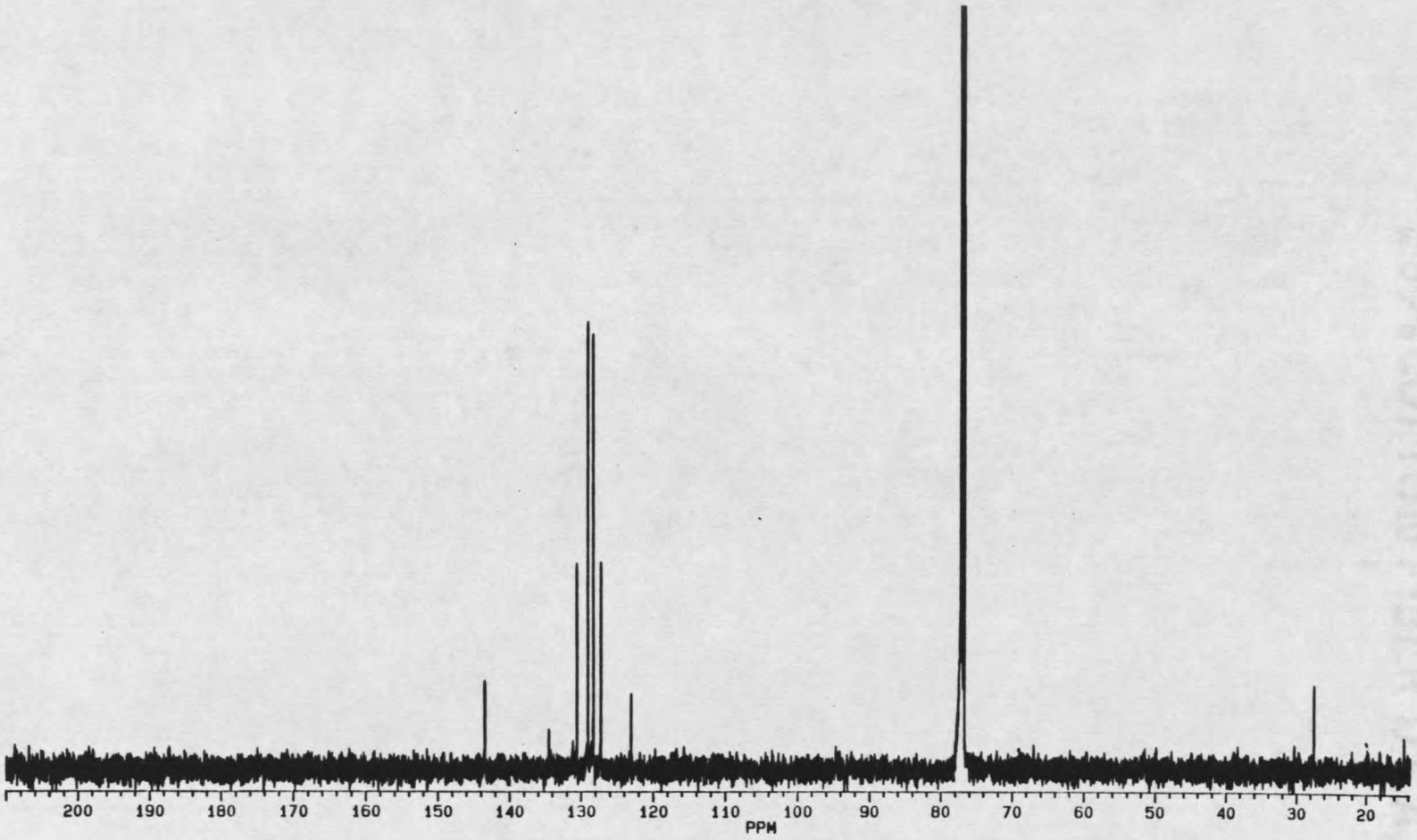


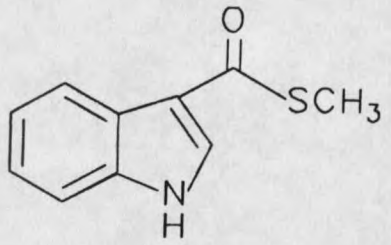
Fig. 44. 125 MHz ^{13}C NMR spectrum of **29** in $CDCl_3$.

Biological Significance and Activity of ***Tedania*** Aromatics

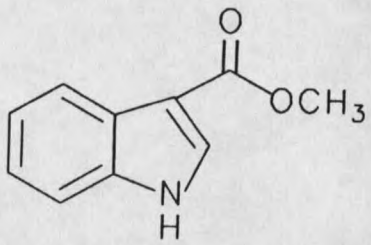
Of the C-3 substituted indoles, only **19** has been reported as a synthetic product (43, 49) and only **23** had previously been isolated as a natural product. 3-(hydroxyacetyl)indole, along with **30**, had been isolated from the marine bacterium, ***Micrococcus*** sp., cultured from a Bermudian ***Tedania ignis*** specimen (31). This strongly implied that **23**, isolated from the sponge extracts, was actually a microbial metabolite.

Indole **23** was also isolated from the marine red alga ***Prionitis lanceolata***. It showed plant growth regulatory effects against lettuce seedlings and may play a role in the chemical ecology of the alga (50).

3-(hydroxyacetyl)indole was shown to be a biosynthetic product of L-tryptophan in the bacterium ***Pseudomonas fluorescens*** (51). This "side chain pathway" utilized the enzyme tryptophan side chain oxidase in a cascade believed to be unique to the bacterium and led to numerous tryptophan side chain metabolites. This lends further evidence towards the microbial origin of the indoles isolated from ***T. ignis***.



30



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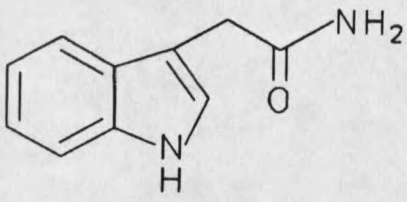

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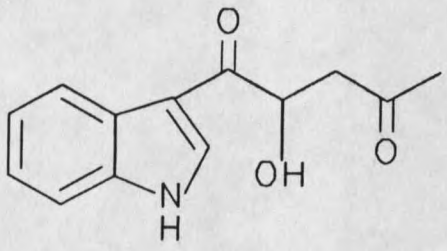

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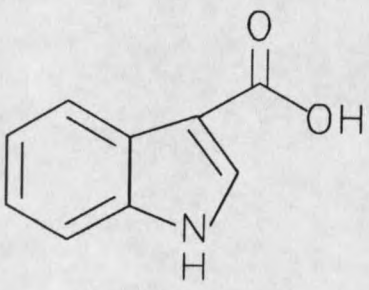
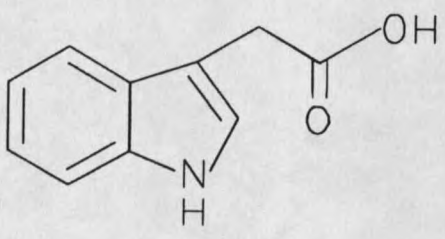


34



35

The simple indoles 20, 31, 32 and 33 have been reported from terrestrial and marine microorganisms (48, 52) as well as sponges (40) and red algae (50, 53).

Compounds 31, 32, and 33 had been shown to act as plant growth regulators (40, 48, 54). This, along with the documented activity of indole acetic acid, 35, as an auxin (55), suggested that the ***Tedania*** indoles might also act as growth regulators. Hence, they were tested in the plant growth assay using lettuce seeds. The results were comparable to those found for other plant regulatory indoles, these being 33, indole 3-acrylic acid, indole 3-acetic acid and caulerpin, a dimer of indole acrylic acid (56). Data for these compounds showed enhanced root elongation over control at $10^{-8}/10^{-9}$M to be 121-122%, 115%, 130% and 121%, respectively (40, 56). At concentrations of 10^{-5}-10^{-10} M, 17, 19 and 23 showed root length at approximately 120% of control. 21 was the most interesting in that at concentrations of 10^{-5} and 10^{-6} M, root elongation was only 10% and 30% of control, respectively, whereas concentrations of 10^{-7}-10^{-10} M showed root length comparable to the other compounds. Thus, 21 was acting more like an auxin, where at high concentrations plant growth was inhibited but at low concentrations growth was stimulated.

Phytotoxicity, brine shrimp and antimicrobial assays were also run on these compounds. 23 showed no activity in any of the three assays. The activity of 17, 19 and 21

is shown in Tables 6, 7 and 8. **21** again proved to be the most potent of this series of compounds.

Table 6. Brine Shrimp Bioassay Data.

Compound	% mortality[a]			
	4 hrs	6 hrs	8 hrs	24 hrs
17	20	20	20	70
19	0	0	8	39
21	0	0	27	91
24	82	100		
25	14	21	43	100
28	0	50	90	100

[a] all compounds tested at 25 ppm
[b] **23, 26, 27** and **29** showed no activity

Table 7. Phytotoxicity Bioassay Data.

plant	compound[a,b]				
	17	**19**	**21**	**24**	**25**
Spotted knapweed	-[c]	-	-	-	-
Leafy spurge	+	-	++	-	-
Yellow nutsedge	+	+	+	+	+

[a] all compounds tested at 1000 ppm
[b] **23, 26** and **28** showed no activity
[c] - : no effects visible
+ : necrotic lesion 0.5-2 mm
++ : necrotic lesion 2-4 mm
+++ : weeping necrotic lesion >4 mm

Table 8. Antimicrobial Bioassay Data.

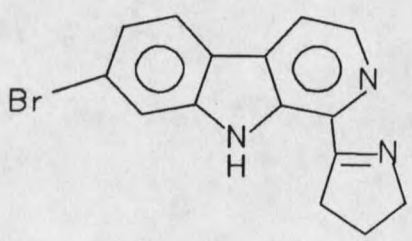
organism	compound[a,b]				
	17	19	21	24	25
S. aureus	1.5 mm	-[c]	4 mm	2 mm	2 mm
B. cereus	-	-	2 mm	2 mm	-
C. michiganensis	-	-	2 mm	NT[d]	1 mm
E. coli	-	-	-	-	-
C. albicans	-	-	-	-	-
G. candidum	2 mm*[e]	1 mm*	-	1 mm*	-
R. solani	-	-	NT	-	-

[a] 17, 19, 21 and 25 were tested at 0.2 mg/disk; 24 was tested at 0.1 mg/disk
[b] 23, 26 and 28 showed no activity
[c] - denotes compound showed no activity
[d] NT denotes organism not tested
[e] *zones exhibited incomplete inhibition

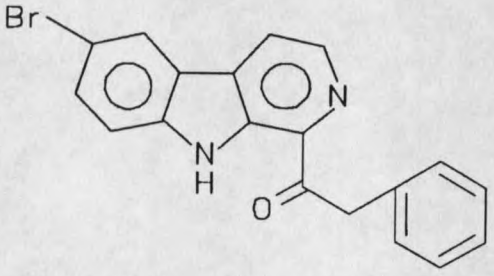
Both 24 and 25 are well known compounds. 1-methyl carbazole (24) is commonly found in cigarette smoke (57), petroleum (58), coal tar and in plant degradation products (44). However, it has never been reported from a marine invertebrate source. It has been shown to be both insecticidal and antimicrobial (59), but unlike 9-methyl carbazole, it was non-mutagenic (60). In house studies showed 24 to be extremely cytotoxic (100% mortality to brine shrimp in less than 6 hours at 25 ppm- Table 6).

Although its activity in the phytotoxicity assay was minimal (Table 7), it was active against the gram positive bacteria ***Staphylococcus aureus***, ***Bacillus cereus*** and ***Corynebacterium michiganensis***. (Table 8).

The β-carboline alkaloids are well known in plants (61). They are not, however, common in the marine environment. The eudistomins are a series of β-carbolines that were isolated from the tunicate ***Eudistoma olivaceum*** (62, 63, 64). Eudistomin G, **36**, and eudistomin S, **37**, are only two examples of this series of compounds.

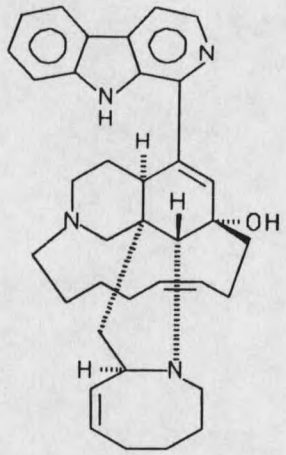


36



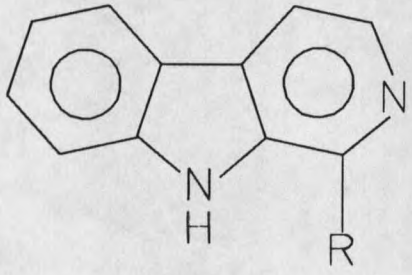
37

The very complex β-carboline, manzamine A, **38**, has been reported from the sponge ***Haliclona*** sp. (65).



38

The only other marine source of β-carbolines was the bryozoan, ***Castaticella hastata*** from which **39**, **40**, **41** and **42** were isolated (66).



39 R= CH_3
40 R= CH_2CH_3
41 R= $CHOHCH_3$
42 R= $CH{=}CH_3$

Harman (39), 40 and pevettine (42) had been previously reported as natural products from terrestrial plants (61, 67).

Although 1-acetyl-β-carboline, 25, has been reported from the terrestrial plant ***Ailanthus malabarica*** (68), this is the first report of its isolation from a marine source. It was found to be cytotoxic in the brine shrimp assay but only minimally active in the antimicrobial and phytotoxicity assays (see Tables 6, 7 and 8).

Both 2-phenylacetamide and 2-phenylacetic acid have been isolated from terrestrial plant sources (69-74) and both are known plant growth regulators (48, 71, 73, 75, 76). 2-phenylacetic acid has also been shown to be phytotoxic (77, 78) and antimicrobial (77, 79, 80). Both compounds are used in penicillin production (81, 82). Microbial sources have also been reported for both compounds. 2-phenylacetamide was isolated from the bacteria ***Actinomyces*** and ***Streptomyces*** (76, 83), while 2-phenylacetic acid was found in the fungi ***Rhizoctonia solani*** (77), ***Aspergillus niger*** (48), ***Ceratocystis fimbriata*** (84) and ***Saccharomyces rouxii*** (85) and clostridia bacteria (86).

The source of 2-phenylacetic acid in ***S. rouxii*** and clostridia was reported to be phenylalanine (85, 86),

which is an end product of the shikimic acid pathway (87). 2-phenylacetamide was also found to be a metabolite of this pathway, its direct precursors being phenylalanine and phenylacetic acid (83).

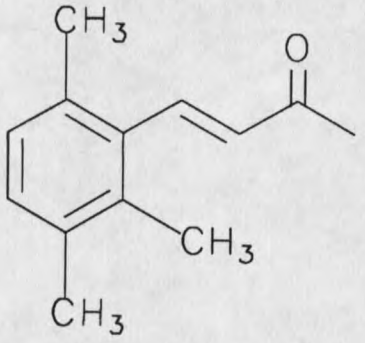
The fact that both compounds are found from microbial sources suggests their origin, in this case, may also be microbial. Their function within the sponge, however, can only be hypothesized. Based on their known plant regulatory ability and phytotoxicity, they may help regulate algal growth on/in the sponge.

Again, this is the first report of **26** and **27** from a marine source. They proved to be inactive in all in house assays. However, because of their already well known plant growth regulatory activity, they were not tested in the plant growth assay.

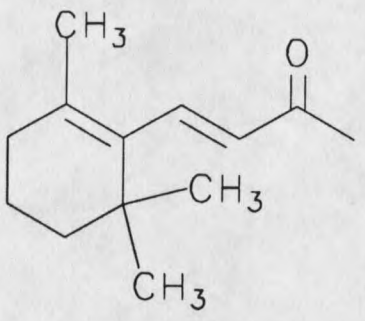
Compound **28** has not been reported from any source. However, 4-(2',3',6'-trimethylphenyl-)but-3-ene-2-one, **43**, a compound differing only in the substitution pattern on the ring, has been reported from the volatile constituents of grape brandies (88), salted and pickled prunes (89) and fruit (90). ^{1}H NMR data comparison between **28** and **43** did little to help confirm the assigned structure for **28**. The ortho protons of **43** were reported as a doublet at δ7.02 (91). This seems to be an incomplete analysis by the

authors since the protons should both appear as doublets. However, it is felt that the nOe experiments run on **28** confirmed the structure.

Compound **43** was also reported as a microbial transformation product when Mikami, et. al. showed that ***Aspergillus niger*** converted β-ionone, **44**, into a mixture containing **43** (91).



43

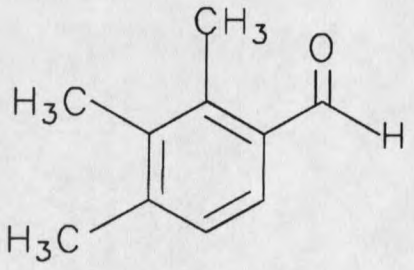


44

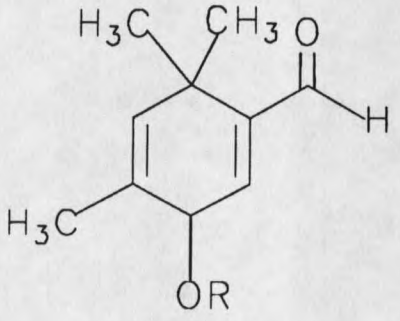
β-ionone is a precursor used in the synthesis of vitamin A. This is interesting when one looks back at the carotenoids **1**, **3** and **4** isolated from ***Tedania***. It appears that **28** may be a degradation product of these carotenoids.

Based on the experimental data of Mikami, it could be theorized that a microorganism is involved in converting the carbocyclic ring found in the carotenes into the aromatic ring of **28** found in the ***Tedania*** carotenoids. It could be that a microorganism is involved in the total synthesis of the carotenoids.

Another origin can be proposed for **28**. If the compound 2,3,4-trimethylbenzaldehyde (**45**) underwent an aldol condensation with acetone, **28** would be the product. **45** has been found in the roots of several plants and is believed to be a rearrangement product of ferulol, **46** (92, 93).



45



46

2-phenylethenyl acetate, **29**, has only been reported as a natural product from one source, the essential oils of the flower ***Gardenia jasminoides*** (94). This is the

first report of its isolation from a marine source. It is believed that 29 is also a metabolite of the shikimic acid pathway. If 29 is hydrolyzed, a vinyl alcohol results, which rearranges to form phenylacetaldehyde. This links 29 to the other previous compounds also believed to be metabolites of the shikimic acid pathway.

Structure Elucidation of Brominated Metabolites

Several compounds were isolated that had simple ^1H NMR spectra, with all signals appearing in the aromatic region. Upon obtaining nominal mass spectral data, it was evident from the isotope patterns that the compounds were highly brominated.

The high resolution mass spectral data for the first compound to be characterized gave a molecular formula of $C_{12}H_6Br_4O_2$, which required eight sites of unsaturation. Mass spectral fragmentation showed consecutive losses of bromine (Fig. 45). The ^1H NMR spectrum (Fig. 46) showed five protons in the aromatic region. The doublet at δ7.80 was coupled to the doublet of doublets at δ7.41 by 2.4 Hz, indicating they were meta protons. The proton at δ7.41 was also ortho coupled to the doublet at δ6.56 by 8.8 Hz. No other coupling was seen for these protons indicating a 1,2,4-trisubstituted aromatic ring. The last two protons

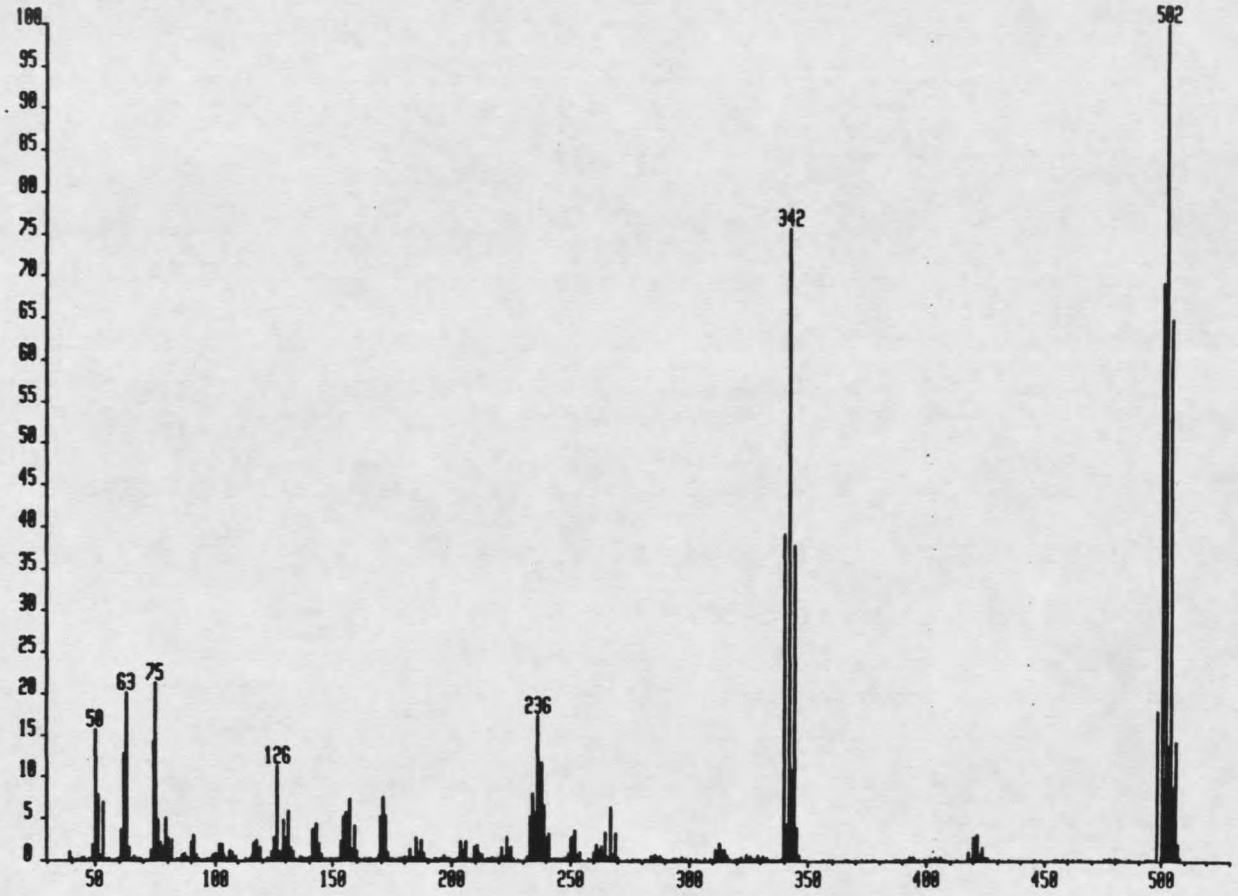


Fig. 45. EI mass spectrum of 47.

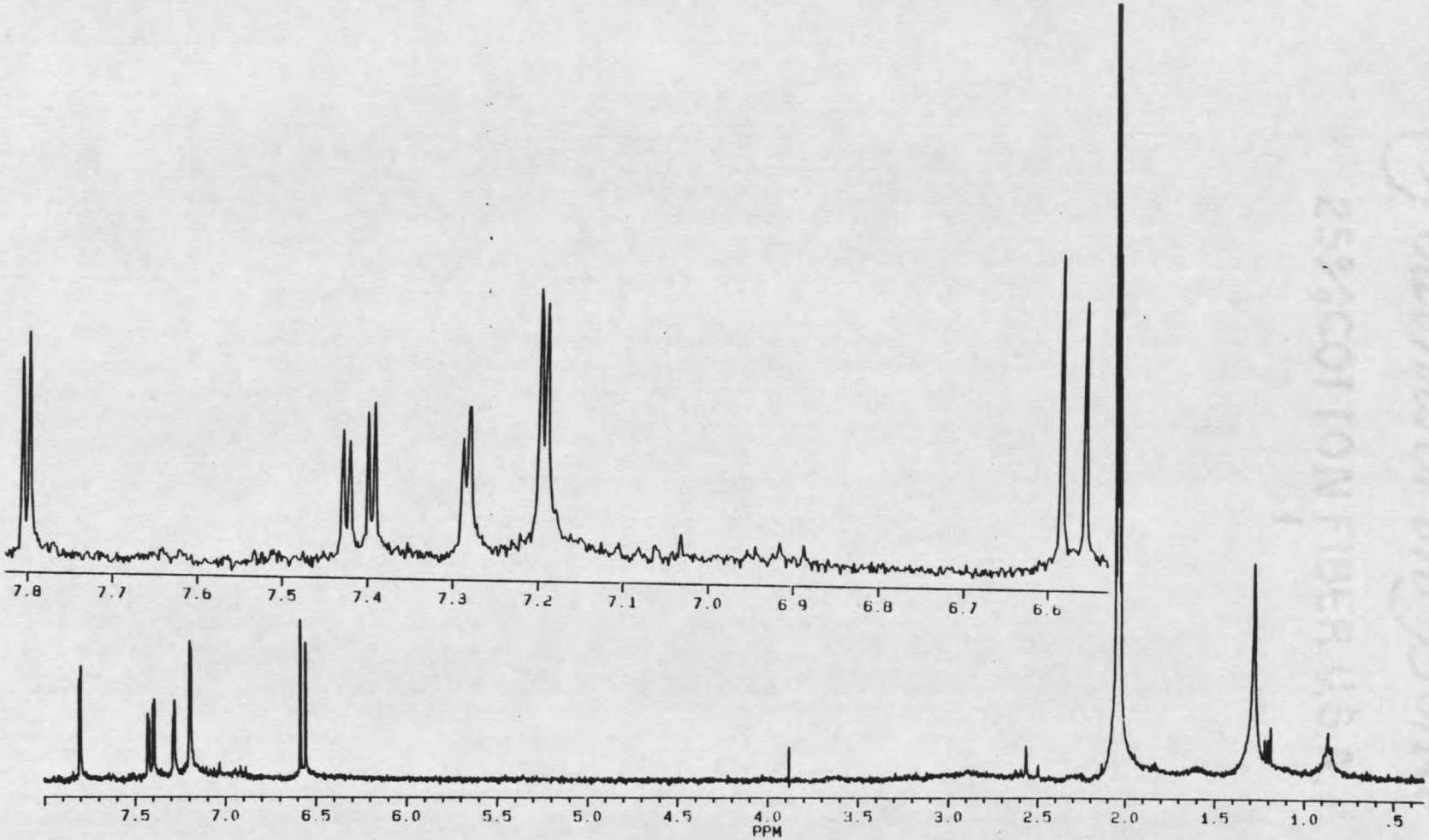


Fig. 46. 300 MHz ^{1}H NMR spectrum of **47** in d_6-acetone.

at δ7.28 and δ7.19 appeared as doublets, meta coupled by 2.2 Hz. This indicated another aromatic ring that was 1,2,3,5-tetrasubstituted. One dimensional decoupling was used to verify these couplings.

The two aromatic rings accounted for all twelve carbons and eight sites of unsaturation, leaving two oxygens, one proton and all the bromines. The presence of a hydroxyl was suspected and to confirm this a TMS derivatization, followed by GC/MS, was performed. The mass spectrum (Fig. 47) showed a molecular ion of 570 (497 + $Si(CH_3)_3$) verifying that one hydroxyl was part of the molecule.

This, in turn, left one oxygen unassigned. Using the oxygen in a ether linkage between the aromatic rings was the only possible conclusion. The four bromines completed the necessary substitution patterns. A ^{13}C NMR spectrum (fig. 48) helped to confirm that all carbons were aromatic and highly substituted with oxygen and bromine.

Placing the substituents at the correct positions around the rings proved interesting. Oxygen is an electron-releasing group and causes a shielding of all protons, although more so to ortho and para protons that to meta protons. Thus, the protons will be shifted upfield in the order of ortho > para > meta. Bromine, on

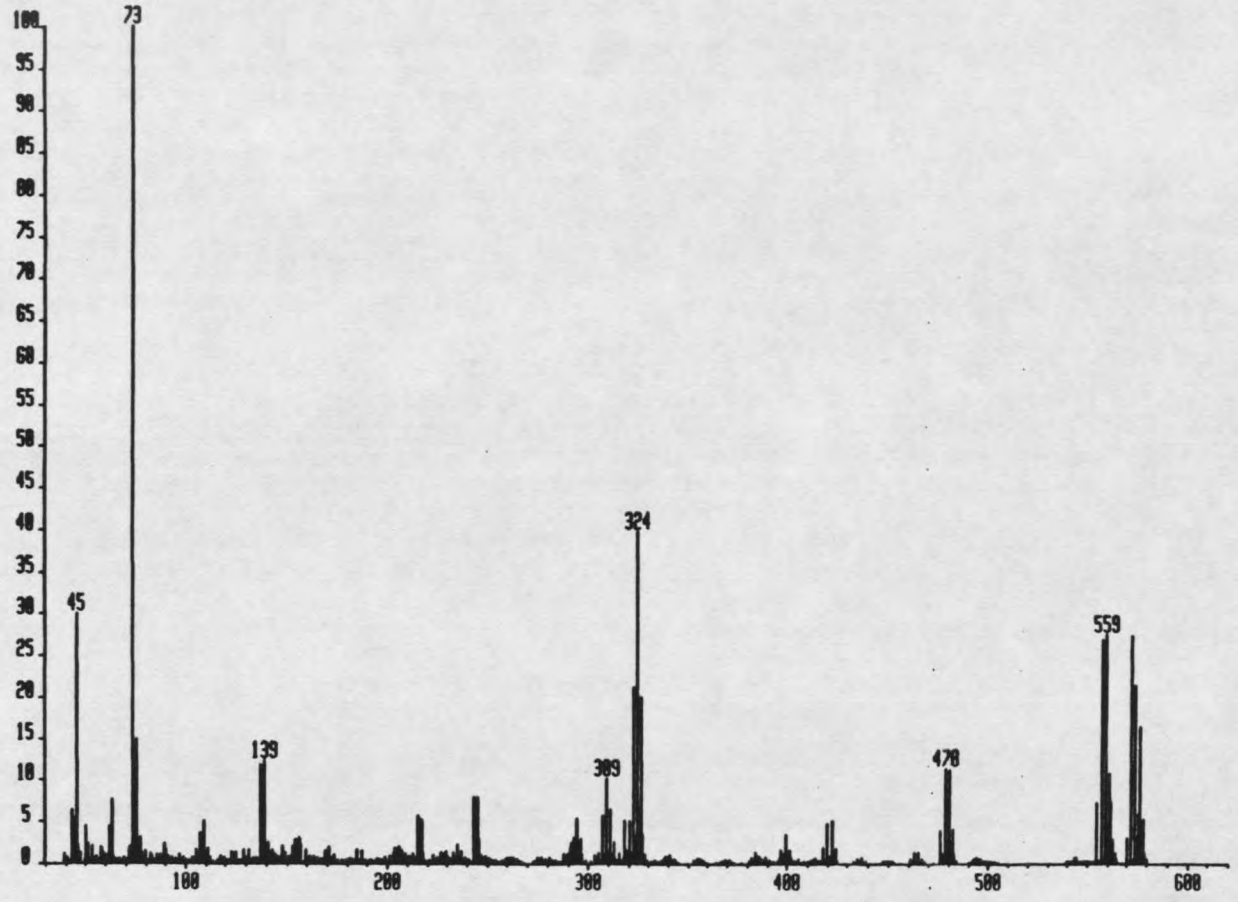


Fig. 47. EI mass spectrum of the TMS derivative of **47**.

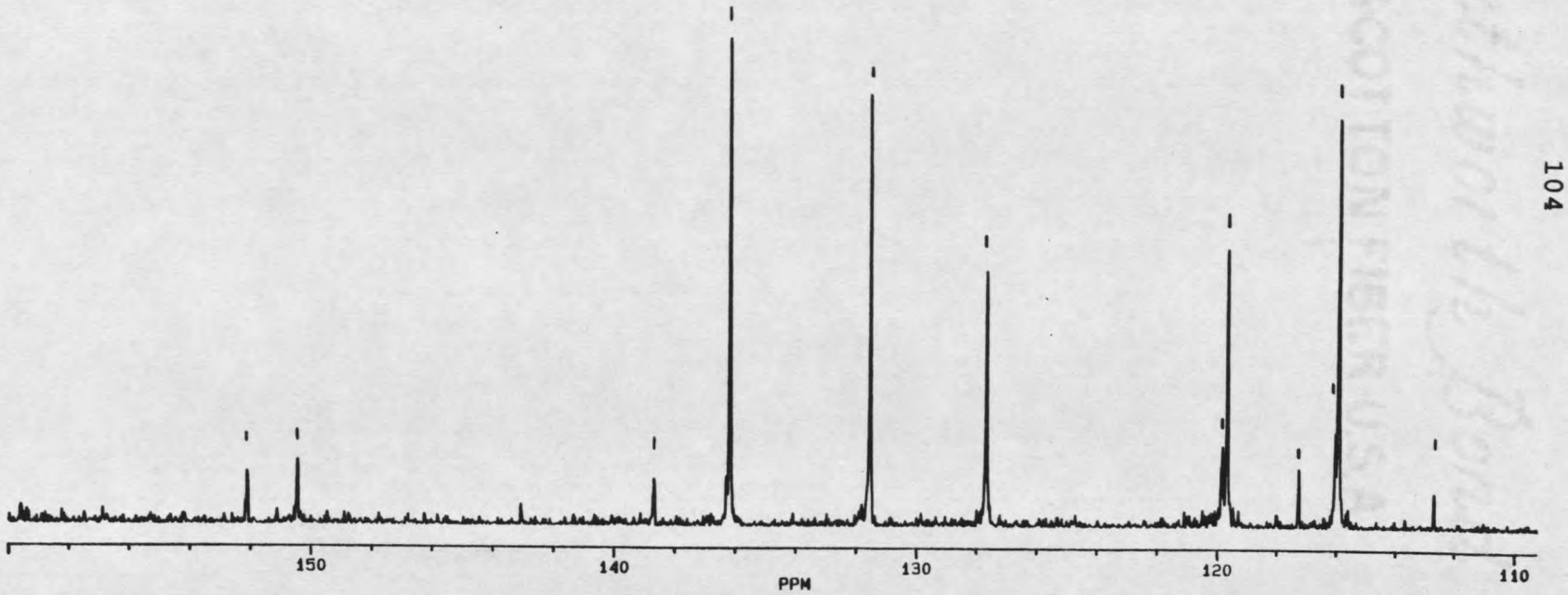
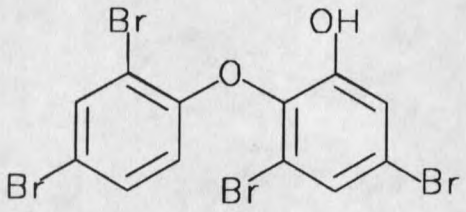


Fig. 48. 125 MHz ^{13}C NMR spectrum of **47** in $CDCl_3$.

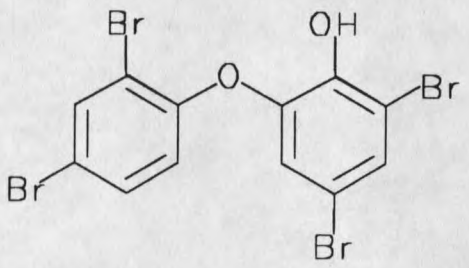
the other hand, causes a downfield shift of ortho protons, an upfield shift of meta protons, and has little effect on para protons.

As stated previously, the first ring had a 1,2,4-trisubstitution pattern. The proton at δ6.56 had to be ortho to an oxygen. The proton at δ7.41, because of its downfield shift, should be ortho to a bromine. The proton at δ7.8 represented a large downfield shift and thus, was most likely situated between two bromines. This completed the first ring.

The two protons on the second ring were meta coupled. The proton at δ7.19 was shifted upfield but not far enough to be influenced only by an oxygen. It had to also be adjacent to a bromine. The shift of the proton at δ7.28 indicated it was probably ortho to bromine but that the bromine's influence was negated somewhat by a para oxygen. Together, this data gave the structure of 2-(2',4'-dibromophenoxy)-3,5-dibromophenol, **47**.



47



48

Another structure, **48**, is possible for this compound. Both compounds had been reported from the sponge, ***Dysidea herbacea***, and Table 9 shows a comparison of their ^{1}H NMR data (95). Carte and Faulkner differentiated the two compounds by converting the phenols to their phenolate ions which causes large upfield shifts to protons ortho and para to the phenolic OH but only small upfield shifts to protons meta to it (95). Comparison of the NMR data showed the compound from ***Tedania*** to match that for **47**.

Table 9. Comparison of NMR data for **47** and **48**.

	47[a]	**47**[b]		**48**[b]	
Carbon #[c]	^{1}H[d]	^{1}H[e]	^{1}H[f]	^{1}H[e]	^{1}H[f]
1	OH				
3	Br			6.82 d	6.97 d
4	7.28 d	7.32 d	7.42 d	Br	Br
5	Br			7.42 d	7.56 d
6	7.19 d	7.19 d	7.18 d	Br	Br
2′	Br				
3′	7.80 d	7.76 d	7.91 d	7.78 d	7.98 d
4′	Br				
5′	7.41 dd	7.26 dd	7.43 dd	7.40 dd	7.58 dd
6′	6.56 d	6.43 d	6.47 d	6.84 d	6.94 d

[a] isolated from ***T. ignis***
[b] isolated from ***D. herbacea***
[c] based on nomenclature for compound
[d] recorded in d_6-acetone
[e] recorded in CCl_4
[f] recorded in d_6-DMSO

The second brominated compound had a molecular formula of $C_{12}H_4Br_4O_3$ and required nine sites of unsaturation, as indicated by HRMS data. Mass spectral fragmentation again showed only consecutive losses of bromine (Fig. 49).

The ^1H NMR spectrum (Fig. 50) revealed three protons, all of which, once again, were aromatic. The protons at δ7.43 and δ7.12 were meta coupled by 1.9 Hz. The proton at δ7.05 was a singlet and hence, had no couplings.

Two aromatic rings were again evident. These accounted for the twelve carbons and eight sites of unsaturation meaning another ring was needed to fulfill the required number of unsaturations. With only oxygens left to form the ring, two structure types could be drawn, a dibenzofuran and a dibenzo-p-dioxin. Since only one proton remained from the molecular formula a dibenzo-p-dioxin seemed more likely. This proton, along with the third oxygen, would form an OH group.

To help verify this, TMS derivatization was again performed. The mass spectrum (Fig. 51) showed a molecular ion at 584 (511 + $Si(CH_3)_3$) indicating that, indeed, only one hydroxyl was present and thus confirming the dibenzo-p-dioxin skeleton.

Using the same reasoning as above, the substitution

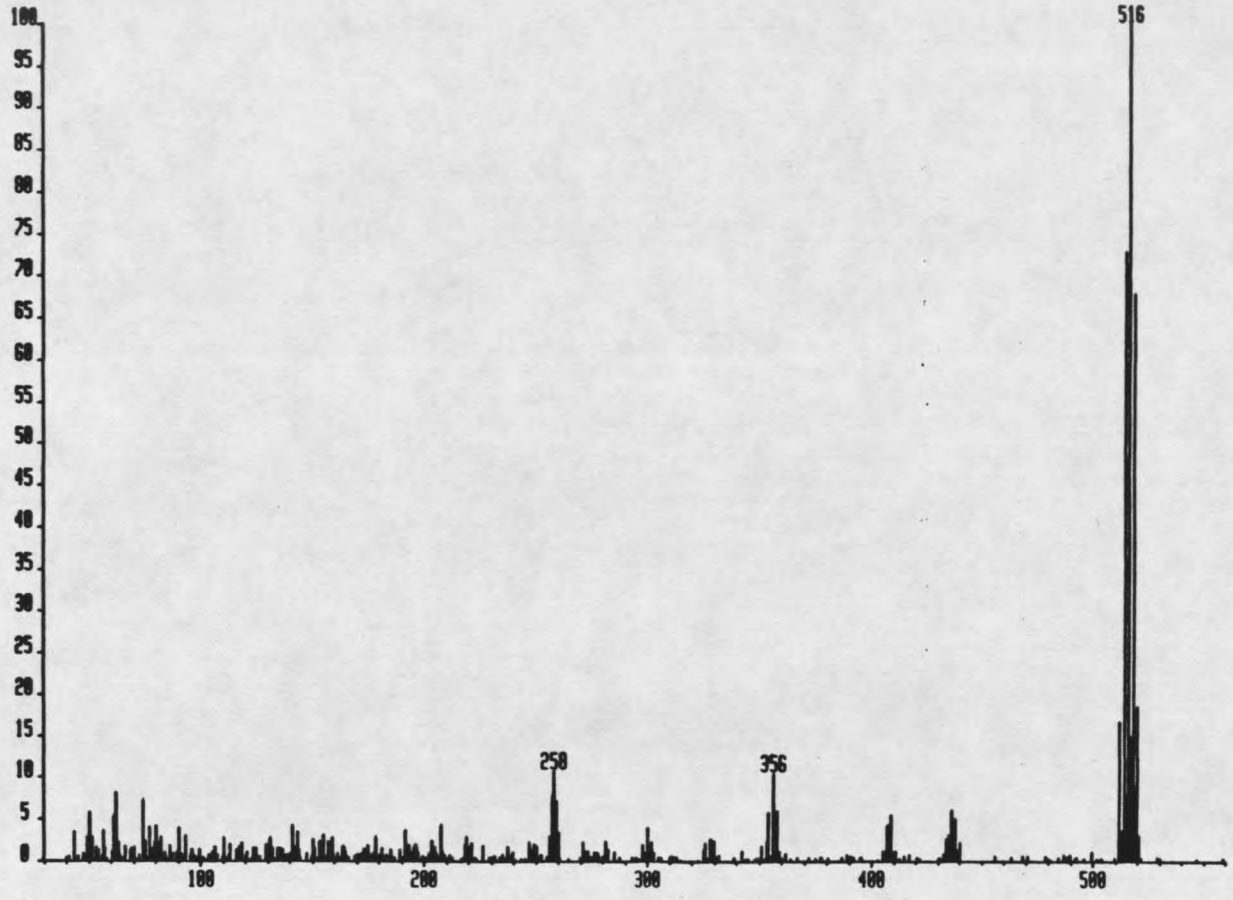


Fig. 49. EI mass spectrum of **49**.

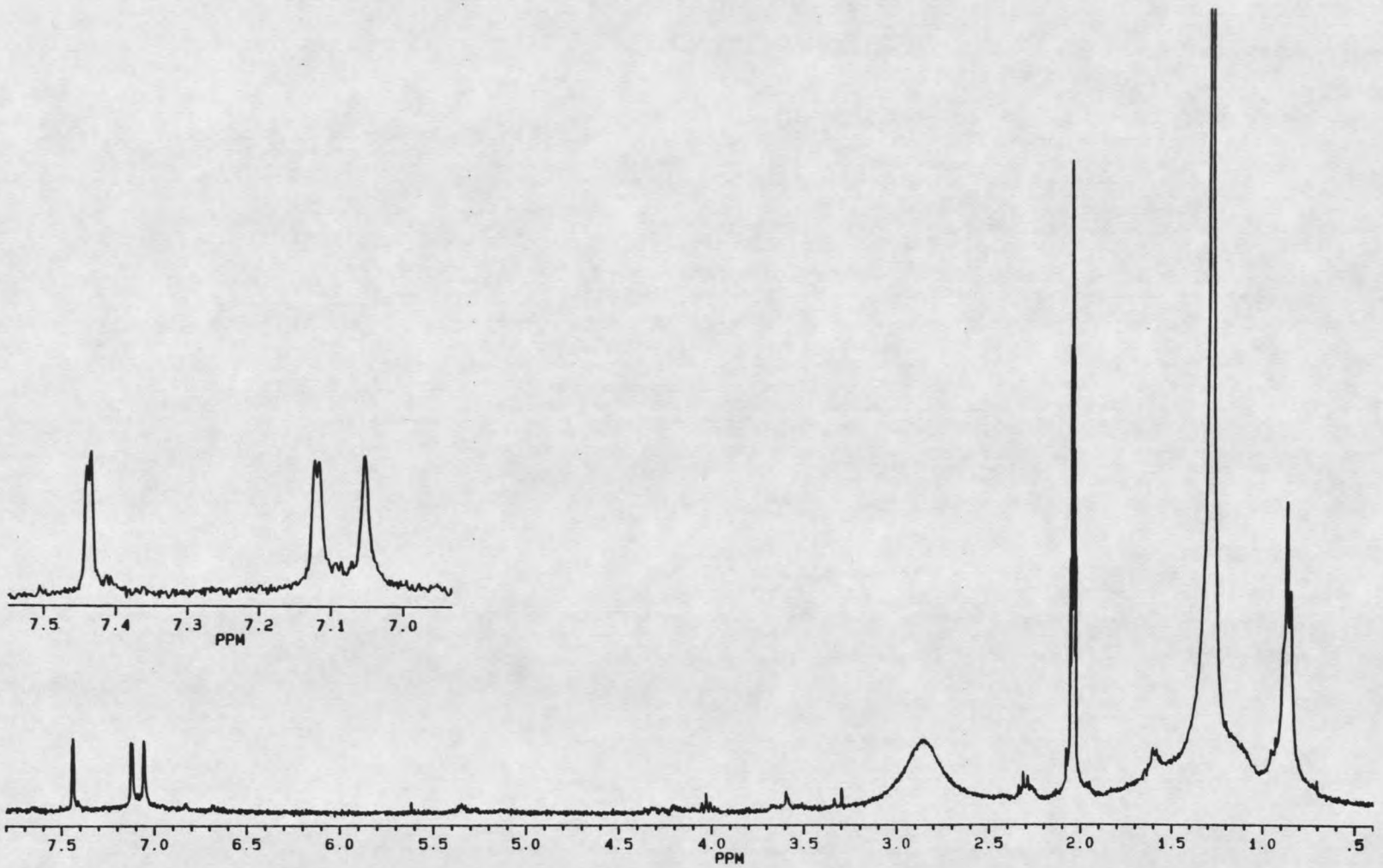


Fig. 50. 300 MHz ^{1}H NMR spectrum of **49** in d_6-acetone.

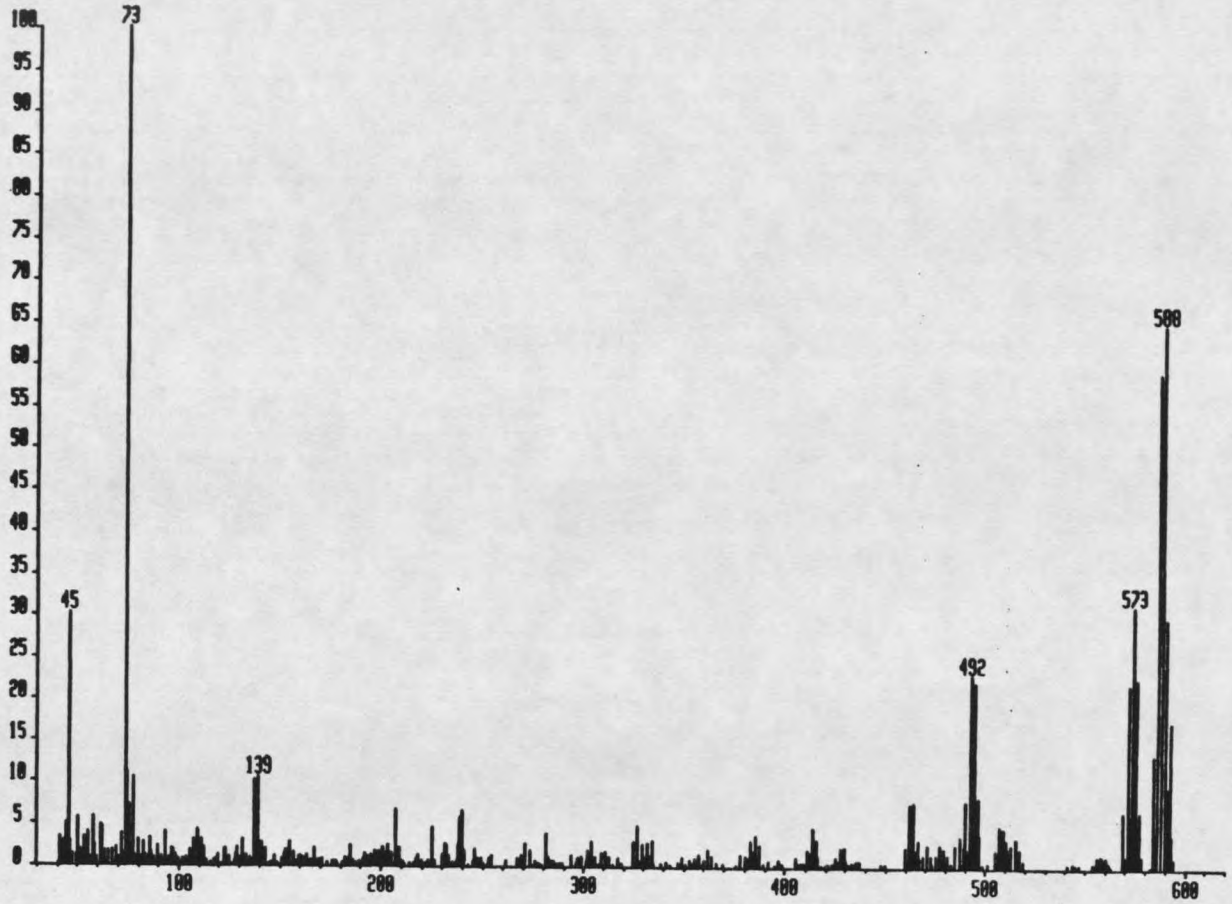
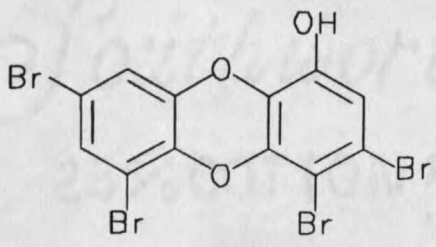


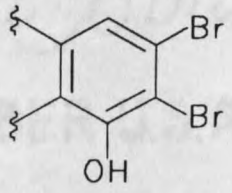
Fig. 51. EI mass spectrum of the TMS derivative of **49**.

patterns for the rings were determined. The proton at δ7.12 was placed ortho to both an oxygen and bromine. The proton at δ7.43 would then be between two bromines. This completed the first ring.

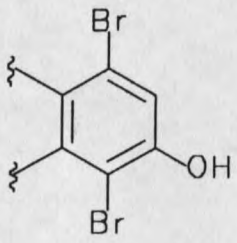
Since the remaining aromatic proton appeared at δ7.05, it was most likely influenced directly by both an oxygen and bromine, thus allowing eight isomers to be drawn. Four of these are represented in **49**.



A



B



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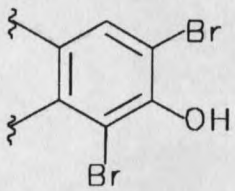
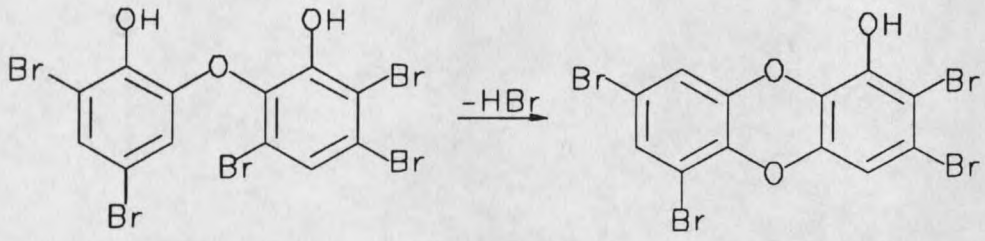


D

49

From the data that could be obtained, the structures were indistinguishable and **49** can only be presented as a

tetrabromohydroxydibenzo-p-dioxin. One structure, however, can be put forward as more probable than the rest based on a brominated diphenyl ether isolated from ***D. herbacea*** from two separate collections (95, 96). Dehydrohalogenation of this ether, **50**, would result in one of the isomers possible for **49** based on proton chemical shifts. Compound **49** will hence be referred to as 1-hydroxy-2,3,6,8-tetrabromodibenzo-p-dioxin.



50 **49**

The last two brominated compounds were isomers that had molecular formulas of $C_{12}H_5Br_3O_3$ and required nine sites of unsaturation, suggesting they were also dibenzo-p-dioxins.

Several attempts to separate the isomers using

adsorption chromatography techniques proved futile. Separation of the isomers was finally achieved by CCC using hexane-CH_2Cl_2-CH_3CN 50:15:35 (descending mode). As the chromatogram (Fig. 52) shows, the isomers were well resolved in less than a hour's time.

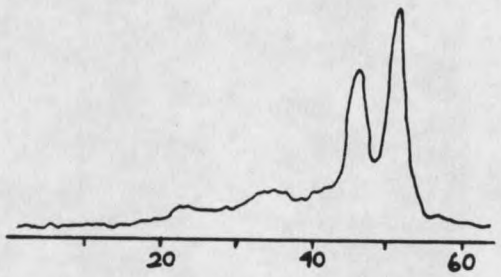


Fig. 52. CCC of 53 and 55; 254 nm; 4 ml/min.

The first isomer collected was 53, the mass spectrum of which showed the consecutive losses of bromine (Fig. 53). Four aromatic protons appeared in the ^1H NMR spectrum (Fig. 54). The doublets at δ7.43 and δ7.12 were meta coupled by 2.4 Hz, while the doublets at δ7.07 and δ6.64 were ortho coupled by 8.9 Hz.

Confirmation of the presence of hydroxyl groups was again achieved by TMS derivatization. The mass spectrum of the derivative (Fig. 55) showed a molecular ion at 506

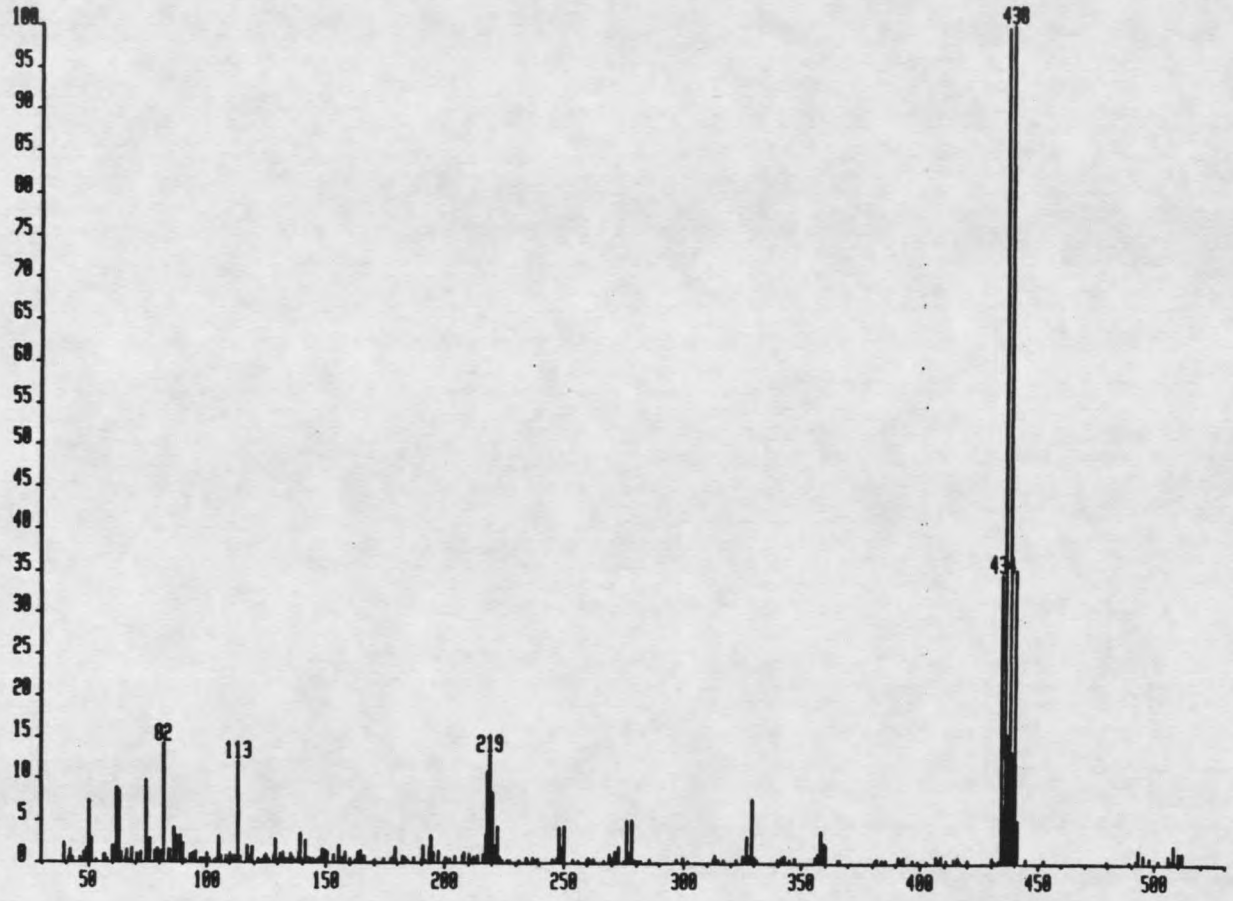


Fig. 53. EI mass spectrum of **53**.

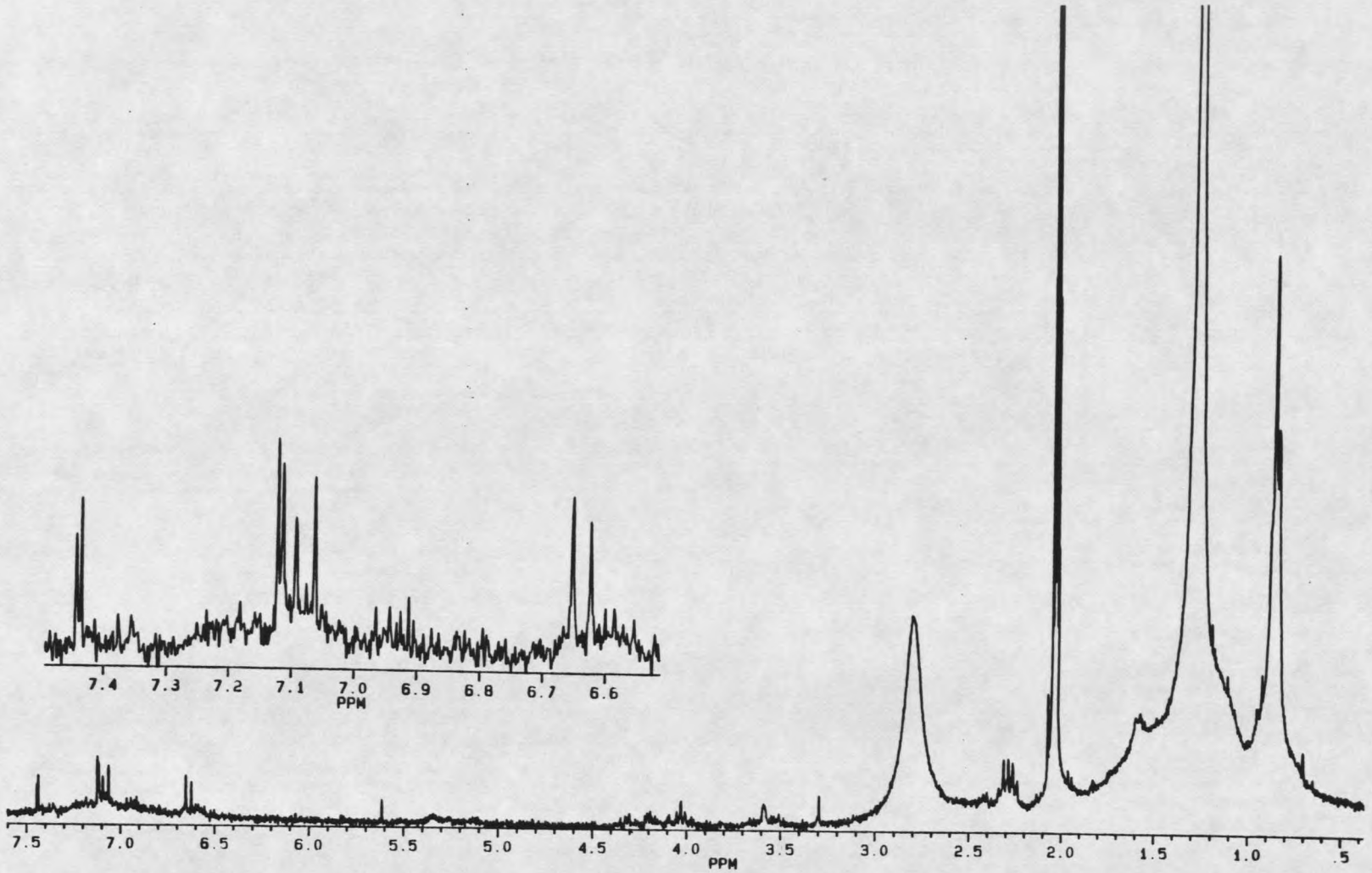


Fig. 54. 300 MHz ^{1}H NMR spectrum of **53** in d_6-acetone.

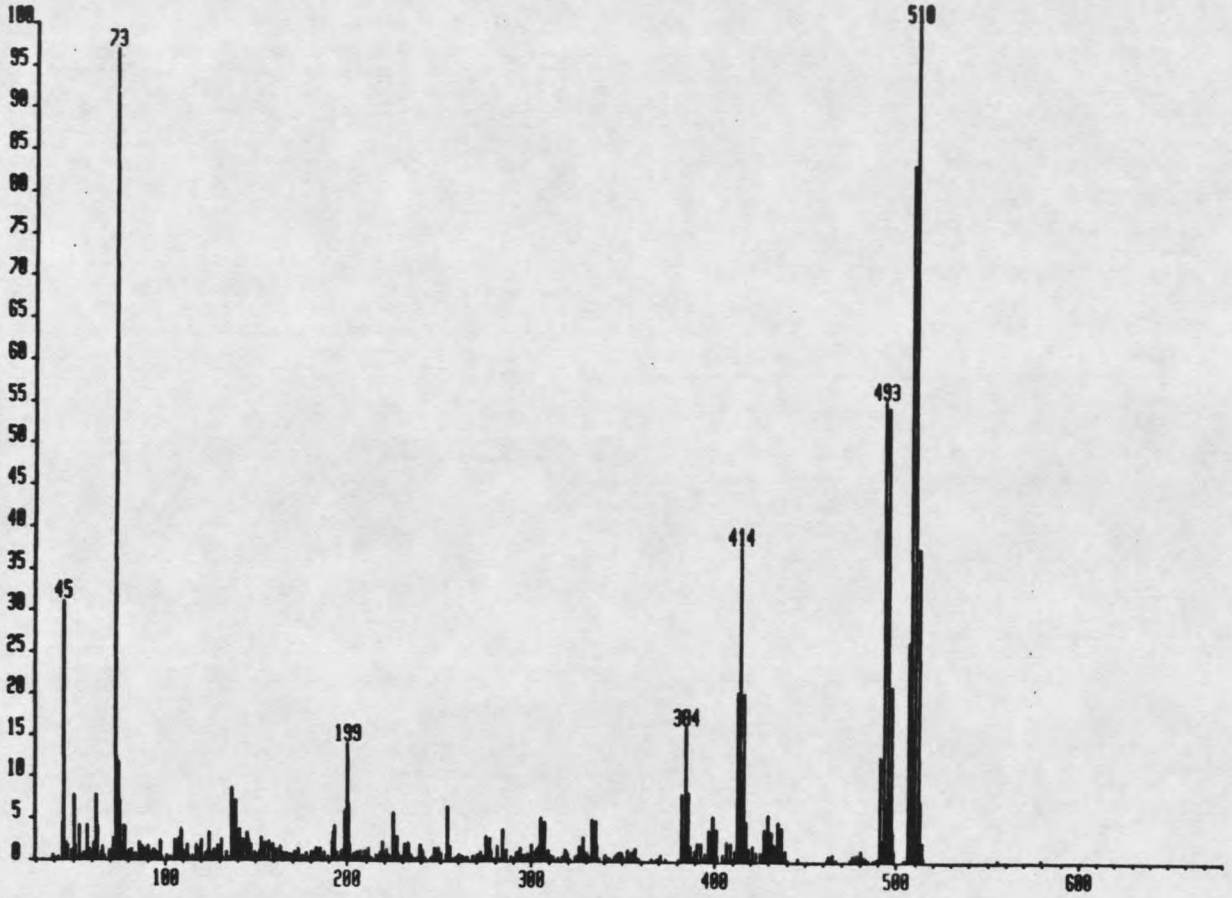
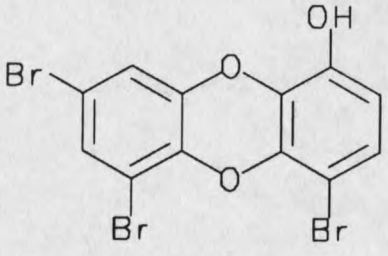


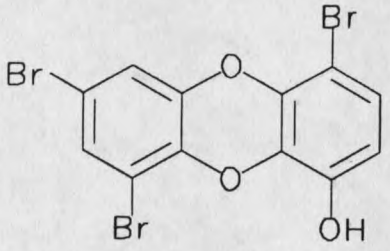
Fig. 55. EI mass spectrum of the TMS derivative of **53**.

(433 + $Si(CH_3)_3$) showing that the molecule had only one hydroxyl group.

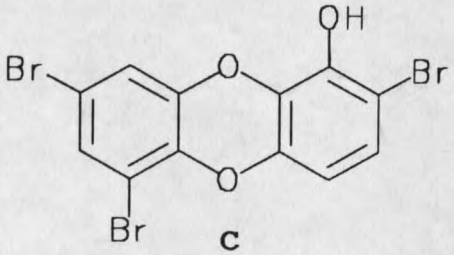
The chemical shifts and couplings of the protons at δ7.43 and δ7.12 matched those of the first ring of compound **49**. The second ring once again had several possibilities. The proton at δ6.64 was, according to its chemical shift, ortho to an oxygen and influenced by a meta positioned bromine. The proton ortho to it (at δ7.07) was ortho to a bromine but still influenced by an oxygen. This allowed the three isomers represented in **51** to be drawn.



A

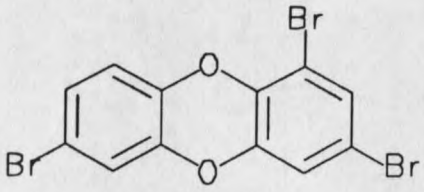


B

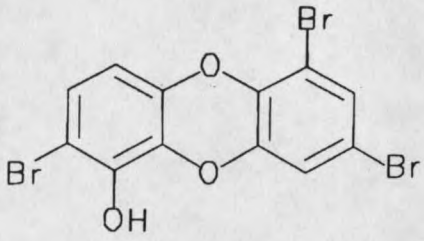


C

Possible differentiation of the isomers was seen when it was realized that dehydrohalogenation of **47** to give **52**, followed by hydroxylation, would result in the formation of **53** (one of the isomers in **51**). Compound **53** is then, most likely, 1-hydroxy-2,6,8-tribromodibenzo-p-dioxin.



52



53

The second isomer, **55**, was collected as the second peak from the CCC and its mass spectrum (Fig. 56) again showed only the consecutive losses of bromine. Once again, TMS derivatization was used to confirm the number of hydroxyl groups. The spectrum (Fig. 57) showed a molecular ion at 506 (433 + $Si(CH_3)_3$) confirming that only one OH was present.

The ^1H NMR spectrum (Fig. 58) showed four aromatic protons. The two doublets at δ6.83 and δ7.42 were meta coupled by 2.3 Hz. Their chemical shifts positioned them

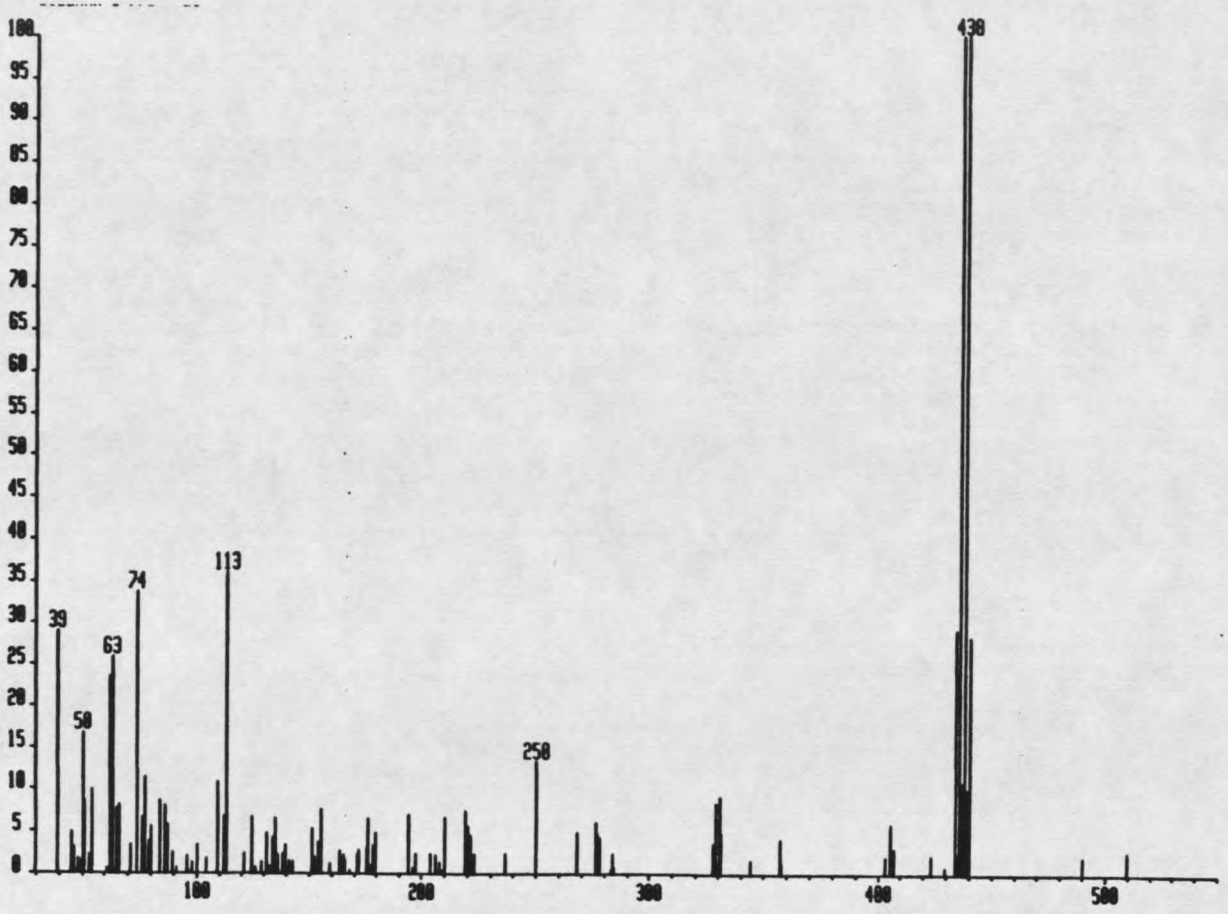


Fig. 56. EI mass spectrum of **55**.

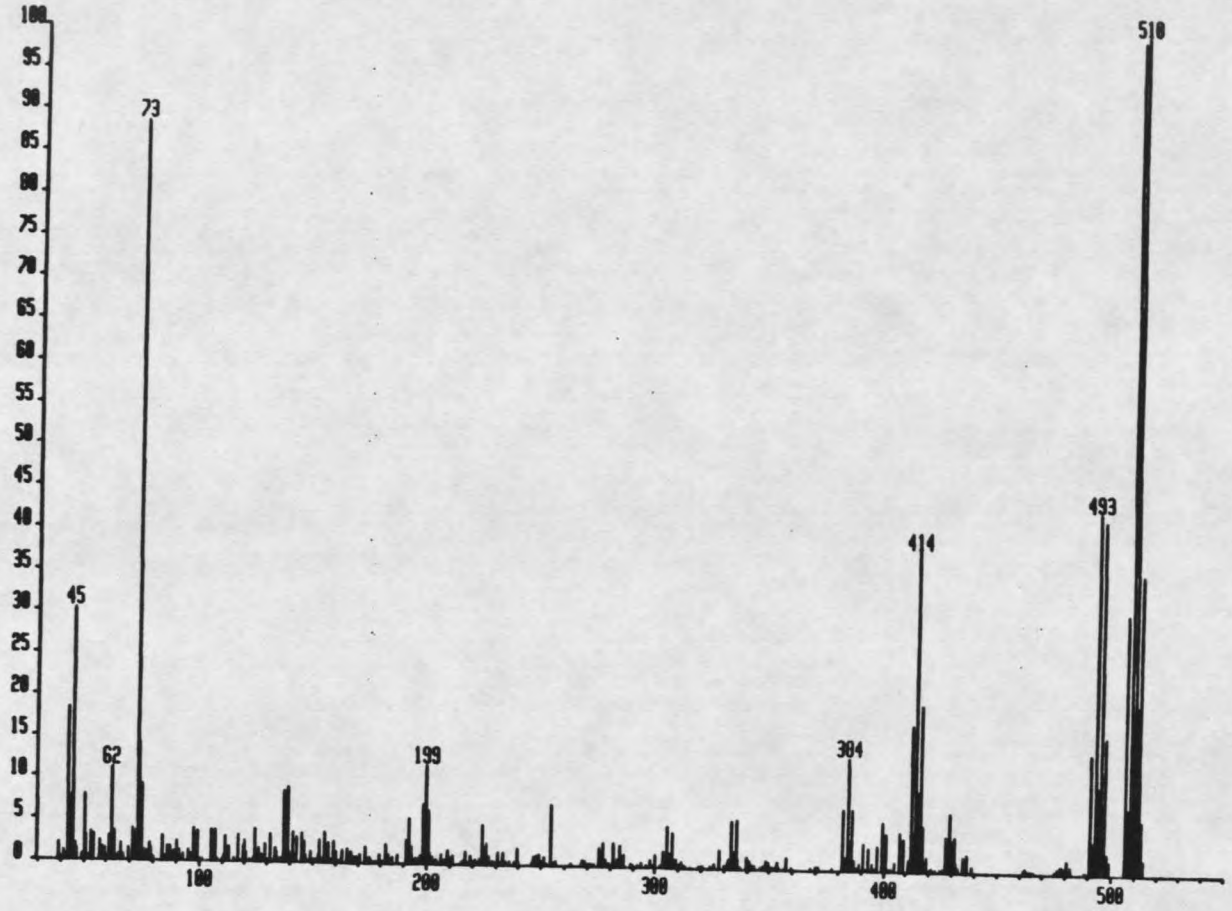


Fig. 57. EI mass spectrum of the TMS derivative of **55**.

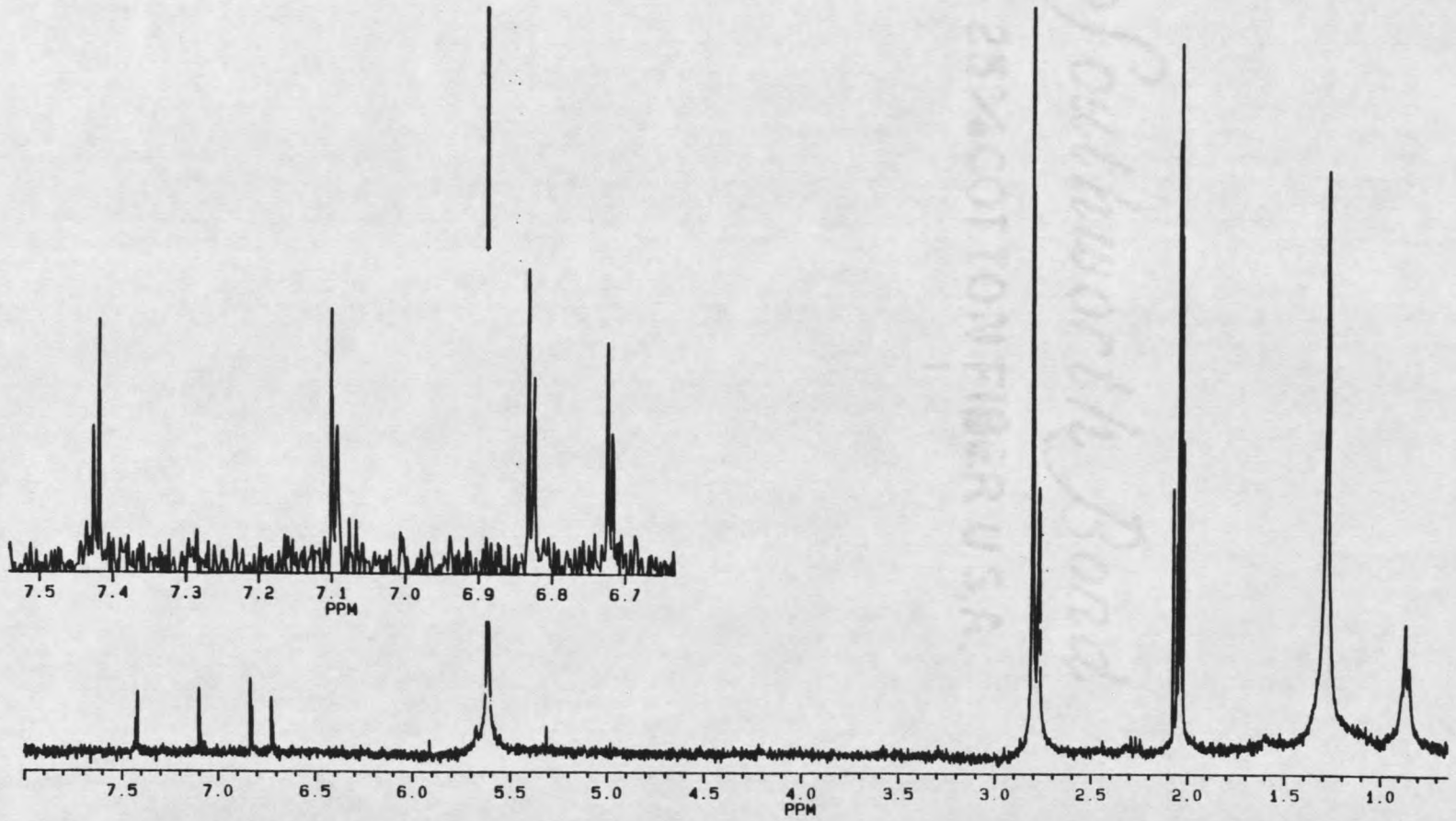
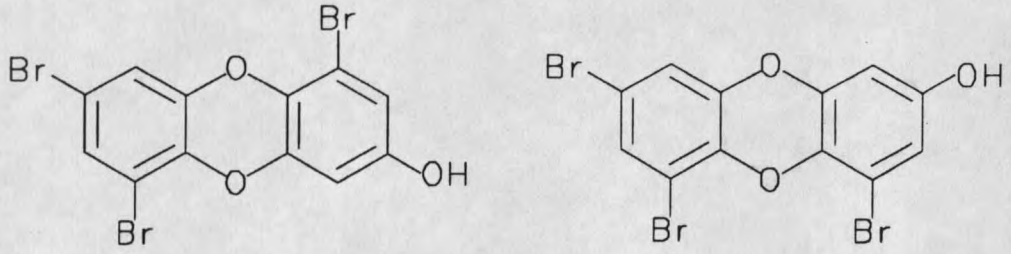


Fig. 58. 300 MHz ^{1}H NMR spectrum of **55** in d_6-acetone.

in the same pattern as seen for the first ring of the previous two dibenzo-p-dioxins. The proton at δ6.83 was, however, shifted further upfield than the proton at δ7.12 indicating that the hydroxyl group that had previously been positioned at carbon-1 was now probably positioned at another carbon (with respect to the proton).

The two doublets at δ6.72 and δ7.09 were also meta coupled by 2.1 Hz. The shift of the δ6.72 proton placed it ortho to an oxygen. The δ7.09 proton, as seen before, should be between an oxygen and bromine. As seen in **54**, this again allowed several isomers to be drawn.



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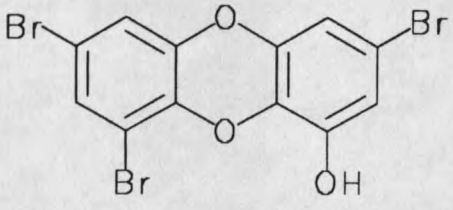
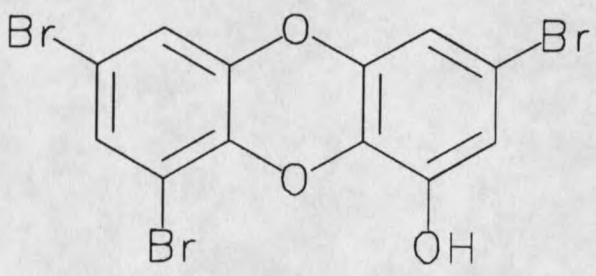


C

54

Once again the isomers could be differentiated based on the formation of the p-dioxin from a known diphenyl ether. Following the same scheme as proposed for the formation of 53, oxidation of 52 at a different carbon would result in the p-dioxin 55. Compound 55 was then 1-hydroxy-3,7,9-tribromodibenzo-p-dioxin.



55

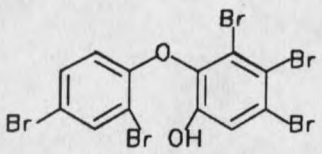
Based on retention times of the GC/MS runs, numerous isomers of the isolated hydroxy brominated compounds were present. Due to the extremely small quantities of these isomers and the fact that no ^{1}H NMR data could be obtained for them, they were not characterized. However, based on molecular weights, compared to the four known compounds, and TMS derivatization data, general conclusions could be drawn. Table 10 gives the data for these isomers.

Table 10. Data for Brominated Isomers

M+	found with:	# of isomers	possible structure
356	49,53	4	hydroxydibromo-dibenzo-p-dioxins
420	47	2	hydroxytribromo-biphenyl ethers
434	49,53	4	hydroxytribromo-dibenzo-p-dioxins (other than 53 and 55)
498	49,53	1	hydroxytetrabromo-biphenyl ether (other than 47)
576	53	1	hydroxypentabromo-biphenyl ether

Biological Significance of Brominated Metabolites

This is the first report of halogenated metabolites from ***Tedania ignis***, although similar compounds have been isolated from other marine invertebrates. As previously stated, the diphenyl ether **47** had been isolated from the sponge ***Dysidea herbacea*** (95). Many other diphenyl ethers, e.g., **56**, **57**, and **58**, were reported from ***Dysidea herbacea***, ***Dysidea chlorea*** and ***Phyllospongia foliascens*** (95), as well as from a green alga, **59** (97) and a sea worm, **60** (98).



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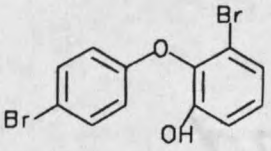

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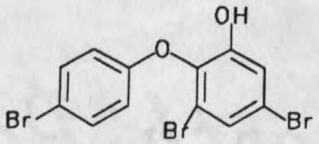

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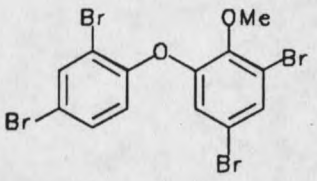

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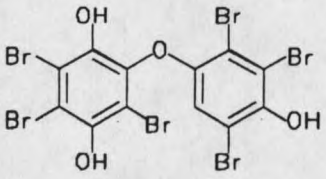
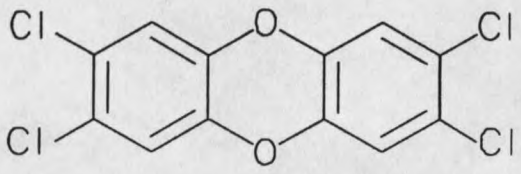


60

In their report of these compounds, Carte and Faulkner suggest the ethers may actually be metabolites of a blue-green alga (cyanobacterium) known to be within the ectosome of the sponge (95). ***Tedania ignis*** is a cleanly collected sponge with no visible growth present on it. However, internal symbiotic microorganisms are most assuredly present. The minute amount of brominated metabolites isolated in this case also helps support the idea of a microbial origin.

The discovery of the brominated dibenzo-p-dioxins was intriguing. The polychlorinated dibenzo-p-dioxins, polybrominated biphenyls and to a lesser extent, polybrominated dibenzo-p-dioxins are environmental

contaminants known to be extremely toxic (99, 100). In fact, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), **61**, is considered one of the most toxic chemicals known to man (101).



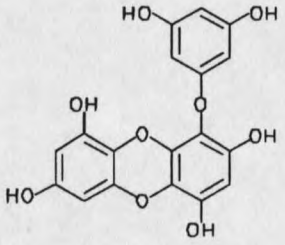
61

Were the brominated compounds found in the sponge merely absorbed contaminants? Several factors led to the belief that they were natural products. First, when found as environmental contaminants, the dioxins and biphenyls are usually isolated together, with the chlorinated compounds being in higher concentrations (100). In this case, no chlorinated compounds were detected.

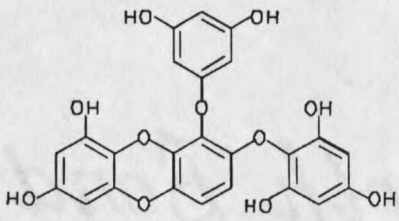
Second, none of the man-made environmental contaminants are hydroxylated, whereas all three of the dioxins isolated from ***Tedania*** contain a hydroxyl group. This, at the least, confirms that the compounds have been metabolized in some manner. A hydroxytetrachlorodibenzo-p-dioxin was found as a metabolite of TCDD in several

studies. The first showed the bacterium ***Pseudomonas testosteroni*** to hydroxylate TCDD after long term incubation (102). However, only 1% of the added material was metabolized. The second study showed the same metabolite of TCDD in the bile of dogs and rats, although in higher concentrations (103, 104).

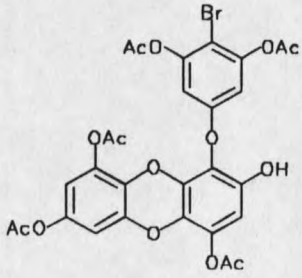
The dibenzo-p-dioxin skeleton is not completely unknown in natural products. Eckol, **62**, and phloroeckol, **63**, are two metabolites isolated from the brown algae ***Ecklonia kurome*** and ***Eisenia arborea*** (105, 106). Although they are not brominated, an acetylated analogue of eckol, **64**, that was also isolated, is monobrominated on a substituent ring (106).



62



63



64

The last argument involves the formation of the brominated dioxins. These types of compounds are known to form by self-condensation of polyhalogenated phenols (107, 108) and by dehydrohalogenation of halogenated diphenyl ethers (95). In fact, Carte and Faulkner synthesized a brominated dibenzo-p-dioxin from one of the isolated brominated diphenyl ethers from ***D. herbacea*** (95). They used the synthetic product as a TLC standard to test for naturally occuring dioxins in the sponge. None, however, were found.

Thus it is believed that the p-dioxins are formed by condensation of two brominated phenols to give a diphenyl ether, followed by further condensation the give the p-dioxin. Both hydroxylation and further bromination may occur after the dioxin is formed and it may be that a microorganism is responsible for the oxidation. In the case of hydroxylation, it is difficult to say what the starting metabolite was since a bromine could be shifted when oxidation occurs (103). This would proceed through an arene oxide as seen in Fig. 59.

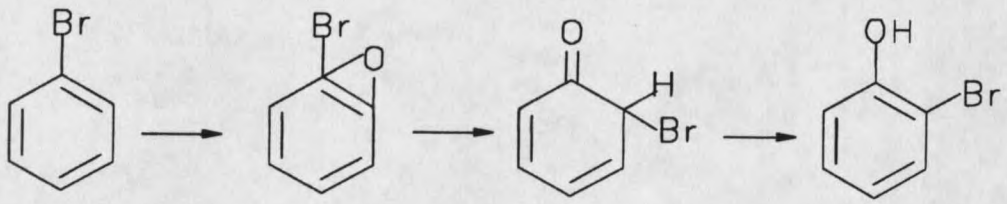


Figure 59. Hydroxylation of Brominated Aromatic.

The purpose of these compounds within the sponge is unknown. One would think they would be toxic due to their structural relationship to the chlorodibenzo-p-dioxins. Their activity, however, should not be extremely potent since it is known from studies performed on the TCDD's that the hydroxylated compounds have a toxicity that is 100 times lower than that of the parent p-dioxins (103).

The compounds were tested in the brine shrimp assay and found to be somewhat cytotoxic. Although none of the compounds were pure, the following results were seen for the mixtures in the brine shrimp assay. Compound **47**, at 25 ppm, caused 50% mortality in 3 hours and 100% mortality in 9 hours. The mixture containing **53** and **55**, at 25 ppm, caused 50% mortality in 2 hours and 100% mortality in 9

hours. Compound **49** was less toxic, causing only 50% mortality after 24 hours.

This difference in toxicity between **49** and the other compounds may be due to several factors. First, the number and position of the bromines on the rings may influence the degree of toxicity. The chlorinated dioxins show this to be true with the 2,3,7,8 tetrachlorodioxin (**61**) being the most potent. The second factor affecting the toxicity of **49** may be the fact that the mixture containing **49** contained the most number of isomers. This may have caused a diluting of the toxic isomer and hence a lessening of its toxicity.

CONCLUSION

This investigation of the secondary metabolites of ***Tedania ignis*** has led to the isolation and characterization of seventeen compounds; three diketopiperazines, four indoles, a carbazole, a β-carboline, four benzenoid compounds, one brominated diphenyl ether and three brominated hydroxydibenzo-p-dioxins. Characterization was completed using the spectroscopic techniques of NMR (^{1}H, ^{13}C, COSY, HETCOR, DEPT, nOe), MS (EI, high resolution, TMS derivatization), IR and UV. Eight of these were novel compounds and only 7, 23 and 47 had previously been reported from a marine source.

As these compounds were isolated, their biological activity was determined. In house assays included antimicrobial, brine shrimp (cytotoxicity), plant growth and phytotoxicity testing. It is intriguing to try to rationalize the purpose of these secondary metabolites based on the results of these assays. One goal of the natural products chemist is the isolation and development of compounds that will be beneficial to man (antibiotics, anticancer, agrochemicals, etc.) and although a metabolite

may be developed in this manner, its true function within its original organism can only be hypothesized.

Two of the compounds isolated, 13 and 23, had been previously reported from a ***Micrococcus*** bacterium cultured from ***Tedania*** and shown to be in a symbiotic relationship with the sponge. This suggested that these compounds, and maybe those structurally related to them (7, 14, 18, 19 and 21) were microbial metabolites. However, this could only be proven by going back to the microbial extracts and isolating these compounds.

The biological purpose of these compounds is unknown. The diketopiperazines were inactive in all in house assays. The indoles, however, did show plant growth regulatory activity. The indole, 23, was especially interesting with its auxin-like activity and it may be that this indole helps to regulate algal growth on or within the sponge. If this compound is indeed a product of a symbiotic microorganism, the relationship between the microbe and the sponge would be of mutualistic benefit. These benefits could include the microorganism keeping the sponge free of algal growth while the sponge provides the microorganism a niche in which to grow.

The isolation of the brominated metabolites from ***Tedania*** was exciting for two reasons. No halogenated

compounds have been previously reported from this sponge and, while **47** has been isolated from ***Dysidea herbacea***, **49**, **53** and **55** are novel compounds.

Many isomers of these metabolites appeared to be present according to GC/MS. Although the major isomers were separated using Sephadex LH-20 and CCC, further separation and characterization were impossible due to extremely small quantities. ^{1}H NMR was used to determine the substitution patterns on the rings and the most likely isomers, via formation from known diphenyl ethers, were presented.

The fact that the dibenzo-p-dioxins are environmental contaminants caused the source of these compounds to be questioned at first. However, as the literature was examined, it was discovered that the chlorinated dioxins were much more common and in a higher concentration. If the ***Tedania*** dioxins were contaminants, where were the chlorinated compounds? For this reason, the brominated dioxins are believed to be true natural products of the sponge or a microorganism associated with the sponge.

Another factor in favor of these compounds being true metabolites is that none of the man-made dioxins are hydroxylated. The only reference to hydroxylated chlorodioxins was as metabolites when organisms (both

micro and macro) were fed TCDD. This shows these compounds are, at the least, metabolized within the sponge.

Two plausible biosynthetic routes to these ***Tedania*** brominated dioxins seem to exist; the condensation of two brominated phenols, followed by hydroxylation of one of the rings or condensation of a brominated phenol with a brominated hydroxyphenol. Either route may proceed through a diphenyl ether intermediate. If the first route is correct, a microorganism may be involved in the hydroxylation.

The function of these metabolites is completely unknown. The chlorinated dioxins are extremely toxic compounds and one can only assume the brominated analogs would be as well. Hydroxylation of the chlorinated dioxins seemed to negate some of their toxicity (104) and hence the hydroxylated brominated dioxins should be less toxic. If these compounds are environmental contaminants, the sponge (or a symbiotic microbe) may hydroxylate them to decrease their toxicity. If a microorganism is responsible for the hydroxylation of the p-dioxins, it could conceivably be isolated and used towards the detoxification of the environmental contaminants.

It would be interesting to go back and recollect

Tedania, as well as other invertebrates in the same area, and look for these brominated dioxins. If the compounds are indeed just metabolized environmental contaminants, other invertebrates, besides ***Tedania***, should have also absorbed them. However, if the compounds are found only in ***Tedania***, they would be true metabolites and a most intriguing find.

EXPERIMENTAL

General

Instrumentation

UV spectra were obtained using a Varian G34 spectrophotometer. IR spectra were obtained with a Nicolet 5DX FT-IR spectrophotometer. Mass spectral data were determined using the VG-MM16F and VG-7070EHF mass spectrometers. NMR spectra were recorded using Bruker AC-300 and Bruker AM-500 spectrometers. Chemical shifts are reported in ppm (δ units) relative to tetramethylsilane ($\delta = 0$). Solvents used were $CDCl_3$, CD_3OD and d_6-acetone, as indicated in the text. Optical rotations were obtained using a Perkin-Elmer 241MC polarimeter with concentrations being reported in g/ml. Melting points were determined with a Fisher-Johns melting point apparatus and are uncorrected.

Chromatography

Gel permeation chromatography was accomplished using the following solid supports: Sephadex LH-20 (25-100 mesh) from Pharmacia Fine Chemicals, Bio-Beads S-X4 (200-400 mesh) and Bio-Beads S-X8 (200-400 mesh) from Bio-Rad Laboratories, silica gel (32-63μm) from Universal Scientific, and C_{18} bonded silica (40μm) from J.T. Baker

Chemical. Fractionation of the eluants was monitored using an ISCO UA-5 (operating at 254 μm) or an ISCO V_4 (variable wavelength) detector. Countercurrent chromatography was performed with an Ito Multi-Layer Coil Separator-Extractor using a 1.6 mm internal diameter coil at a speed of 800 rpm and flow rate of 4 ml/min.

Bioassay Procedures

Antimicrobial Assay

Compounds were tested against seven organisms including ***Staphylococcus aureus***, ***Bacillus cereus***, ***Cornynebacterium michiganensis***, ***Escherchia coli***, ***Candida albicans***, ***Geotrichium candidum*** and ***Rhizoctonia solani***. A single organism was inoculated into a tube of sterile distilled water and allowed to incubate at room temperature for 24 hours. An aliquot from the tube was spread on a Petri dish containing either tryptic soy agar or mycological agar until a smooth lawn was formed. Sterile paper disks, 1/4" in diameter, were impregnated with the compound to be tested and allowed to air dry. The disk was placed on the surface of the dish. The cultures were allowed to incubate at room temperature for 24-48 hours. Activity was measured by a halo of non-growth that appeared around the disk. The zone of

inhibition was measured from the edge of the disk to the edge of the halo and was reported in mm.

Brine Shrimp Assay

The brine shrimp assay is a simple, nonspecific test used to indicate the cytotoxicity of a compound. It is especially useful in the rapid evaluation of fractions (109). Approximately 10 to 15 brine shrimp eggs were placed into a 5 ml test tube containing 4 ml of Instant Ocean. The eggs were incubated at room temperature for 48 hours at which time the number of hatched nauplii were counted. A few drops of Baker's yeast solution was then added. An appropriate amount of compound, in 20 µl of ethanol, was injected into the tube. The surviving number of nauplii were counted at 4, 6, 8 and 24 hours.

Phytotoxicity

A nicked leaf assay was conducted using spotted knapweed (***Centaurea maculosa***), leafy spurge (***Euphorbia esula L.***) and yellow nutsedge (***Cyperus esculentus***). The compound to be tested was dissolved in enough 2% aqueous ethanol solution to give the desired concentration and applied to the nicked surface of a leaf in a 5 µl droplet. Two test leaves were run for each compound along side a control leaf. A 5µl droplet of 2% aqueous ethanol was used with the control. The leaves were placed on

moistened filter paper and sealed in a Petri dish. After 72 hours, a comparison was made between the control leaf and the test leaves. The leaves were observed for signs of necrosis and chlorosis.

Plant Growth Assay

The compound to be tested was made up in an aqueous solution ranging from 10^{-5} to 10^{-10} M. Five ml of each dilution was placed in a separate covered Petri dish. Twenty lettuce seeds (Great Lakes iceberg) were placed on a screen supported on a Teflon spacer. The dish was placed in a light box and root length was measured after 48 hours (53).

Crude Extraction of *Tedania ignis*

Tedania ignis was collected in the shallow waters off the coast of Bermuda in the fall of 1979 and stored in acetone. After storage at -5°C, the acetone:water was removed by filtration and the acetone evaporated in vacuo. The solids were ground in methanol which was then filtered. The methanol was removed, leaving an aqueous residue which was combined with the water from the first filtration. The solids were soaked twice overnight in CH_2Cl_2. Each time, the CH_2Cl_2 was removed by filtration. The aqueous residue was combined with the CH_2Cl_2 and a

solvent partition was performed. The CH_2Cl_2 was removed and the aqueous layer was partitioned thrice more with fresh CH_2Cl_2. This partition gave the organic crude extract (86.36 g) and the aqueous crude extract (506.5 g).

The organic crude was then separated using a solvent partitioning scheme. The crude extract was dissolved in 90% MeOH:H_2O and extracted three times with hexane. The hexane fractions were combined and the solvent removed in vacuo, leaving the hexane soluble extract (72.0g; 83.4% of organic crude). The water in the aqueous layer was increased to 20% and the resulting solution extracted three times with CCl_4. The CCl_4 fractions were combined and the solvent removed in vacuo leaving the CCl_4 soluble extract (5.60g; 6.5% of organic crude). The water in the aqueous layer was increased again to 35% and the resulting solution extracted with $CHCl_3$. The $CHCl_3$ fractions were combined and the solvent removed leaving the $CHCl_3$ soluble extract (7.80g; 9.0% of organic crude). The MeOH was removed from the aqueous layer which was then extracted several times with ethyl acetate. The EtOAc layer yielded an extract of 0.40g (0.5% of organic crude). The H_2O was freeze-dried and gave an extract weighing 0.56g (0.65% of organic crude). Figure 60 shows the extraction of ***T. ignis*** in schematic form.

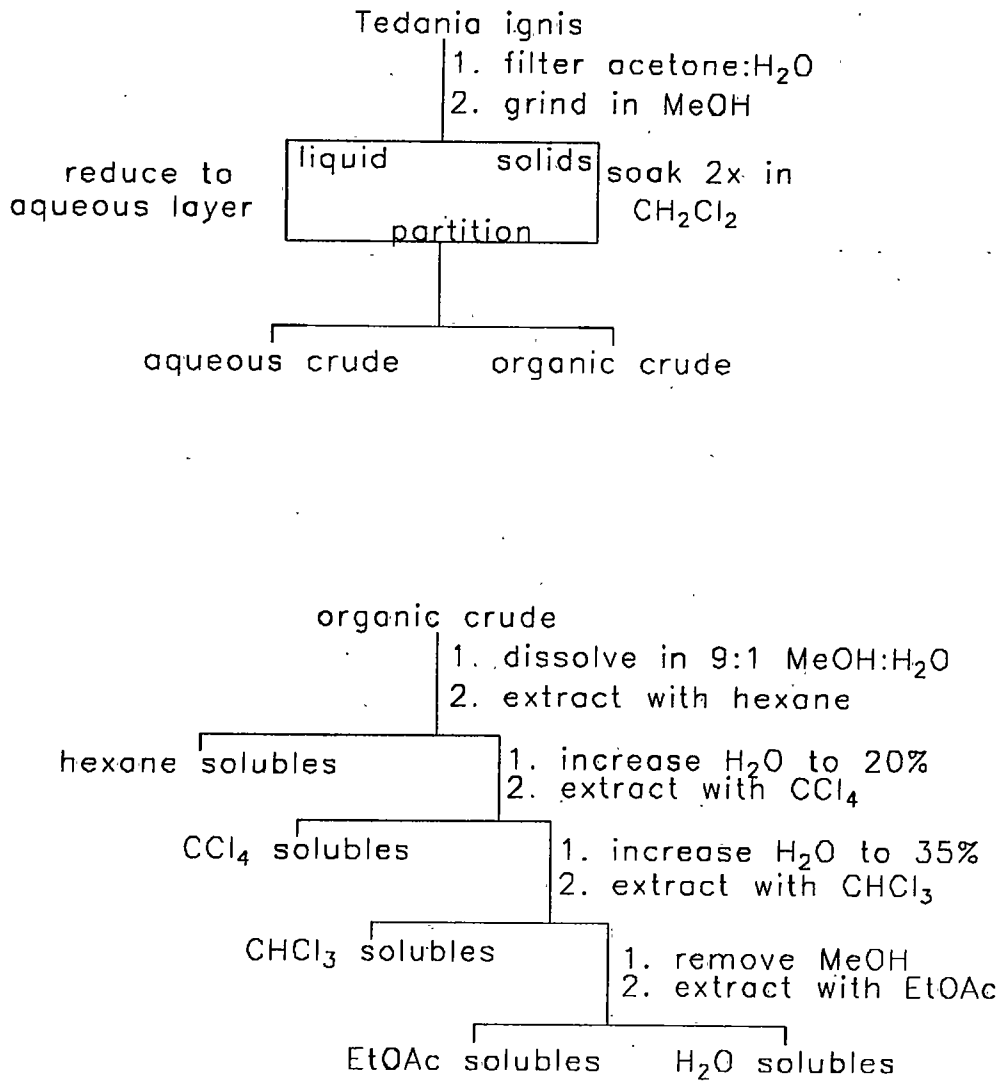


Figure 60. Extraction scheme for ***Tedania ignis***.

Isolation of *Tedania* Metabolites

Separations were, at first, based on cytotoxicity of the fractions in the brine shrimp assay. However, as chromatographies proceeded, those fractions having interesting NMR spectra were further investigated.

The CCl_4 crude from the solvent partition was chromatographed on a BioBeads S-X4 column (94 x 4.5 cm) using 4:3:1 hexane-CH_2Cl_2-EtOAc. Seven fractions were obtained with fractions 3-7 exhibiting brine shrimp activity. The sixth fraction was permeated through a Sephadex LH-20 column (180 x 2.5 cm) using 1:1 CH_2Cl_2-MeOH. The separation yielded six fractions of which 2-6 were brine shrimp active. Fraction four was 25 and fraction six was 24.

The fourth fraction from the BioBeads S-X4 column was also permeated through the above Sephadex system. Six fractions were obtained with fractions 1-4 showing brine shrimp activity. Fractions two and three were applied separately to the CCC using 25:34:20 $CHCl_3$-MeOH-H_2O (descending). Fraction two yielded five fractions, the first of which was further purified on silica gel (35 x 2.5 cm, low pressure) using 20% CH_3CN in CH_2Cl_2 to give 28. The third fraction gave six fractions from the CCC, the first of which was further separated on silica (low

pressure) using 5% and 20% CH_3CN in CH_2Cl_2 to yield **17**.

Fractions five and seven from the BioBeads S-X4 column were also both applied separately to a Sephadex LH-20 column (1:1 MeOH-CH_2Cl_2). Fraction five yielded six fractions, all of which showed brine shrimp activity. The second fraction was further purified using the above CCC system. This yielded seven fractions. Fractions three and five were the diketopiperazines **13** and **7**, respectively. The sixth fraction from this Sephadex LH-20 column was applied to another Sephadex LH-20 column (158 x 2 cm) using 4:1 MeOH-CH_3CN. The third and fifth fractions were **47** and **49**, respectively. The fourth fraction was a mixture of compounds that were further separated on the CCC using 50:15:35 hexane-CH_2Cl_2-CH_3CN (descending). The second and third fractions were **53** and **55**, respectively.

Fraction seven from the BioBeads S-X4 column yielded six fractions from the Sephadex LH-20 column. Fractions one and two were combined on the basis of NMR analysis. These two fractions appeared to contain a diketopiperazine. Further separation on a Sephadex LH-20 column (137 x 2.0 cm) using 1:1:1 CH_2Cl_2-isopropanol-methyl t-butyl ether gave six fractions. The third fraction was the diketopiperazine **14**.

Fraction four from the first Sephadex LH-20 column was separated using the CCC with the aqueous system described above. Fraction seven from this separation was **27**.

The $CHCl_3$ crude from the solvent partition was permeated through the BioBeads S-X4 column (4:3:1 hexane-CH_2Cl_2-EtOAc) as a first separation. Six fractions were obtained with fractions two, three, five and six being active in the brine shrimp assay. Both the fifth and sixth fractions were applied separately to a Sephadex LH-20 column using 1:1 CH_2Cl_2-MeOH. The fifth fraction yielded six fractions, of which fractions three, five and six were separately applied to the CCC using 25:34:20 $CHCl_3$-MeOH-H_2O (descending). Fraction six yielded **23**. Fraction five yielded nine fractions, of which both fractions two and three were further separated on silica (low pressure) using 20% CH_3CN in CH_2Cl_2 to give **21** and **19**, respectively. Fraction eight was **26**. The separation of fraction three from the Sephadex LH-20 column on the CCC yielded six fractions, the first of which was purified on silica (low pressure) with 5% CH_3CN in CH_2Cl_2 to give more of **17**. Fraction four from the Sephadex LH-20 column was applied directly to silica (low pressure) using 20% CH_3CN in CH_2Cl_2 and yielded **29**.

The sixth fraction from the BioBeads S-X4 column, upon separation on the Sephadex LH-20 column, yielded nine fractions. Only fraction one showed no brine shrimp activity. The sixth fraction was applied to the CCC using the above system and yielded more of **26**.

The fifth fraction was permeated through a BioBeads S-X8 column (137 x 2.0 cm) using 3:2 CH_2Cl_2-cyclohexane. This yielded four fractions. Fraction one, when applied to the CCC using the aqueous system as above, gave seven fractions. Fraction five was the diketopiperazine **14**. Figure 61 gives a schematic representation of the separation of the ***Tedania*** metabolites.

Characterization of Compounds

Cyclo(L-Pro-L-Phe), 13

Yield: 5.9 mg from 5.6 g of CCl_4 solubles; $[\alpha]_D$= -117.7° (c= 0.0059, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ7.15-7.35 (5H, m), 5.60 (1H, br s), 4.25 (1H dd, J= 10.5, 3 Hz), 4.05 (1H, t, J= 7 Hz), 3.60 (2H, m), 2.75 (2H, dd, J= 14.5, 10.5 Hz), 2.30 (2H, m), 2.0 (2H, m); ^{13}C NMR ($CDCl_3$) δ169.3, 165.1, 136.0, 129.3 (2 carbons), 129.1 (2 carbons), 127.5, 59.2, 56.2, 45.4, 36.9, 28.4, 22.6; EIMS m/z (%) 244 (M^+, 47), 153 (35), 125 (100), 91 (57).

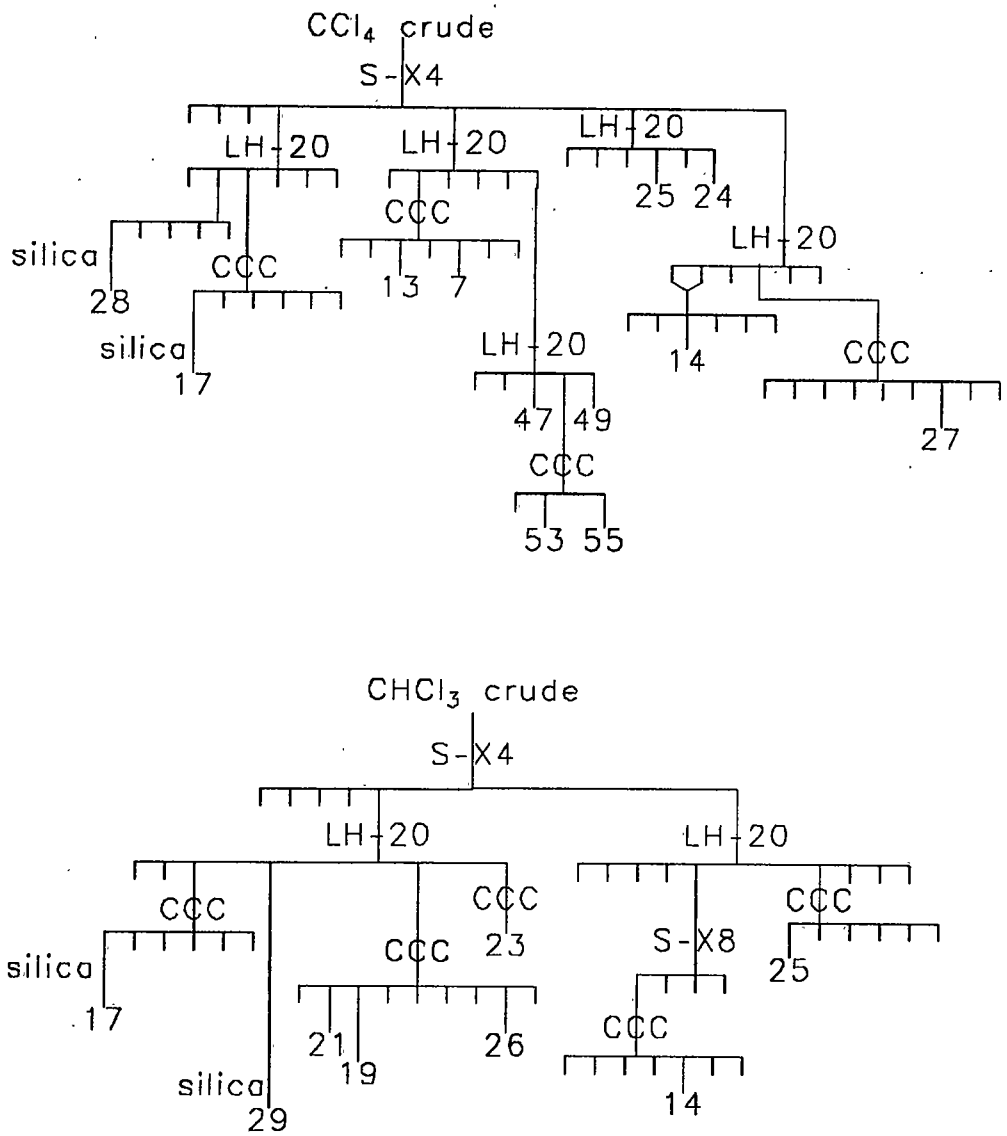


Figure 61. Separation scheme for ***Tedania ignis*** metabolites.

Cyclo(L-Pro-L-Val), 7

Yield: 4.8 mg from 5.6g of CCl_4 solubles; $[\alpha]_D$= -113.7° (c= 0.0047, $CHCl_3$); ^1H NMR ($CDCl_3$) δ5.85 (1H, br s), 4.08 (1H, t, J= 7 Hz), (3.92 1H, br s), 3.50-3.70 (2H, m), 2.62 (1H, m), 2.35 (2H, m), 1.80-2.10 (2H, m), 1.08 (3H, d, J= 7 Hz), 0.90 (3H, d, J= 7 Hz); ^{13}C NMR ($CDCl_3$) δ169.8, 164.9, 60.5, 58.8, 45.1, 28.6, 28.5, 22.4, 19.2, 16.1; EIMS: m/z (%) 196 (M^+, 1), 154 (90), 125 (29).

Cyclo(L-Pro-L-thioPro), 14

Yield: 4.6 mg from 13.4 g of CCl_4 and $CHCl_3$ organic solubles; white crystalline solid; $[\alpha]_D$= -114.3° (c= 0.0014, $CHCl_3$); IR ν_{max} ($CDCl_3$) 1659, 1423 cm^{-1}; ^1H NMR ($CDCl_3$) δ4.75 (1H, d, J= 10 Hz), 4.5 (1H, d, J= 10 Hz), 4.45 (1H, t, J= 7 Hz), 4.2 (1H, t, J= 8 Hz), 3.55 (2H, m), 3.45 (1H, dd, J= 12, 7 Hz), 3.35 (1H, dd, J= 12, 7 Hz), 2.35 (1H, m), 2.15 (1H, m), 1.95-2.05 (2H, m); ^{13}C NMR ($CDCl_3$) δ23.2, 27.8, 32.5, 45.3, 48.5, 60.5, 62.8, 164.2, 165.8; HRMS: m/z (%) 212.0648 (M^+, 100, $C_9H_{12}N_2O_2S$ requires 212.0620), 138 (17), 124 (20), 87 (15).

Synthesis of Cyclo(L-Pro-L-thioPro), 14

The first step in the synthesis of cyclo(L-Pro-L-thioPro) involved protecting one amino acid with BOC-ON

according to the procedure by Itoh (33). To 0.133 g (1.0 mM) of L-thioproline was added 0.2735 g (1.1 mM) BOC-ON, 10 ml of 15% aqueous acetone and 200 µl triethylamine. The mixture was stirred for 2 hours at room temperature. The mixture was evaporated and the aqueous layer washed with EtOAc. The aqueous layer was then acidified with 1M HCl and extracted with CH_2Cl_2. The crude product yield was 0.1792 g (64%).

The next step involved reacting the BOC-protected amino acid with the methyl ester of the second amino acid. The method followed was that by Nitecki, et. al. and was known to proceed without racemization (34).

The BOC-thioproline (0.1792 g) was dissolved in 10 ml CH_2Cl_2 and 140 µl triethylamine. L-proline methyl ester hydrochloride (0.1648 g, 1.0 mM) was added along with 0.1920 g (1 mM) N-ethyl-N'-(3-dimethyl-aminopropyl)-carbodiimide hydrochloride. The mixture was stirred overnight at -5 °C. The solution was washed sequentially with equal volumes of H_2O, 1N citric acid, 5% $NaHCO_3$ and H_2O. The organic layer, which contained the crude product, was evaporated to dryness.

The t-BOC-dipeptide methyl ester was dissolved in 10 ml of formic acid and stirred at room temperature for 2 hours. The formic acid was removed, in vacuo, and the

dipeptide ester formate was dissolved in 5 ml toluene and 25 ml sec-butanol. The mixture was refluxed for two hours and the solvents removed by rotary evaporation.

The crude diketopiperazine was purified on a Sephadex LH-20 column (158 x 2.0 cm) using 4:1 MeOH-CH_3CN. Upon removal of solvents, fraction five was a white crystalline solid whose spectral data matched that of the natural product. The yield for the second reaction sequence was 63%. The overall yield of the synthesis was 38%. $[\alpha]_D$= -112.8° (c= 0.0019, $CHCl_3$); mp 168-170 °C. Spectral data for the synthetic product exactly matched that of the natural product.

Synthesis of Cyclo(L-tyr-L-thioPro), 16

The synthesis of cyclo(L-tyr-L-thioPro) was performed by following the same procedure as that for the synthesis of cyclo(L-Pro-L-thioPro). At first, L-thioproline was protected with BOC-ON and then reacted with L-tyrosine methyl ester HCl. However, in the final step, the dipeptide ester formate would not cyclize. It was decided to try to reverse the amino acids by protecting L-tyrosine with BOC-ON and reacting it with L-thioproline methyl ester HCl.

The L-tyrosine was protected with BOC-ON by again

using the procedure of Itoh (33). To 0.186 g (1.0 mM) of L-tyrosine was added 0.2719 g (1.1 mM) BOC-ON, 15 ml of 50% aqueous acetone and 750 µl triethylamine. The percent of water in the acetone and the amount of triethylamine used had to be adjusted in order to increase the solubility of the tyrosine. In the amounts used previously (15% aqueous acetone and 200 µl of triethylamine), the tyrosine appeared to be completely insoluble. The above adjustments allowed the tyrosine to dissolve within 1/2 hour with stirring. The mixture was stirred for a total of 3 hours at room temperature because of this solubility problem. The mixture was then evaporated, washed with EtOAc, acidified and extracted with CH_2Cl_2. Yield of the crude L-tyrosine-BOC was 0.2379 g (85.3%).

The L-thioproline methyl ester hydrochloride had to be synthesized. This was done following a procedure on methyl esterification of amino acids by Rachele (110). Two mM (0.2674 g) of L-thioproline was suspended in 20 ml of 2,2-dimethoxypropane. Two ml of 36% v/v HCl were added and the mixture was stirred at room temperature for 18 hours. The solvents were then evaporated, leaving the crude L-thioproline methyl ester hydrochloride.

The linking and cyclization of the amino acid again

followed the procedure by Nitecki (34). The L-tyrosine-BOC was dissolved in 10 ml CH_2Cl_2. To this were added the L-thioproline methyl ester, 750 µl triethylamine and 0.1950 g (1mM) of N-ethyl-N'-(3-dimethylaminopropyl)-carbodiimide hydrochloride. The mixture was stirred at 2°C overnight. The solution was washed sequentially with H_2O, 1N citric acid, 5% $NaHCO_3$ and H_2O, after which the organic layer was evaporated, leaving the t-BOC dipeptide methyl ester. This was then dissolved in 10 ml formic acid and stirred at room temperature for 2 hours. The formic acid was evaporated and the dipeptide ester formate was then dissolved in 5 ml toluene and 25 ml sec-butanol. The solution was refluxed for two hours and the solvents removed by rotary evaporation.

The crude diketopiperazine was chromatographed on a Sephadex LH-20 column using 4:1 MeOH-CH_3CN. Fraction 5 contained the product but was not completely clean. This fraction was then run on the CCC using 25:34:20 $CHCl_3$-MeOH-H_2O (descending). The first fraction contained the pure cyclo(L-tyr-L-thioPro). Product yield was 7.5 mg. Overall yield was 2.8%. $[\alpha]_D$= -2.2° (c= 0.00045, EtOH); mp 128-130°C. HRMS: m/z (%) 278.0717 (M^+, 6, $C_{13}H_{14}N_2O_3S$ requires 278.0725), 172 (30), 107 (100); ^1H NMR ($CDCl_3/CD_3OD$) δ6.9 (2H, d), 6.7 (2H, d), 4.9 (1H, d), 4.26

(1H, t), 4.2 (1H, d), 4.1 (1H, m) 3.1 (2H, m), 2.9 (1H, dd) 2.25 (1H, dt); ^{13}C NMR ($CDCl_3/CD_3OD$) δ166.1, 164.5, 156.2, 130.7 (2 carbons), 125.5, 115.6 (2 carbons), 61.0, 56.7, 46.7, 37.7, 32.9.

6-(indol-3-yl)-5-methylhept-3(E),5(E)-diene-2-one, 17

Yield: 8.3 mg from 5.6 g CCl_4 solubles; yellow oil; UV λ_{max} (EtOH) 224 nm (ε= 12,800), 290 (ε= 8,300); IR ν_{max} ($CDCl_3$) 3475, 1653 cm^{-1}; HRMS: m/z (%) 239.1289 (M^+, 80, $C_{16}H_{17}NO$ requires 239.1310), 224 (73), 196 (100), 181 (42), 167 (13), 154 (16), 130 (16), 117 (16), 89 (3); ^1H NMR (d_6-acetone) δ10.3 (1H, br s), 8.0 (1H, d, J = 15.5 Hz), 7.4 (1H, d, J= 8 Hz), 7.2 (1H, d, J= 8 Hz), 7.13 (1H, s), 7.10 (1H, t, J= 8, 7 Hz), 6.90 (1H, t, J= 8, 7 Hz), 5.6 (1H, d, J = 15.5 Hz), 2.19 (3H, s), 2.16 (3H, s), 1.73 (3H, s); ^{13}C NMR ($CDCl_3$) δ199.4, 146.8, 142.2, 135.9, 127.7, 127.3, 126.7, 123.1, 122.1, 119.7, 119.7, 113.8, 111.1, 28.0, 24.4, 20.8.

4-(indol-3-yl)but-3(E)-en-2-one, 19

Yield: 3.3 mg from 7.8 g $CHCl_3$ solubles; yellow oil; UV λ_{max} (EtOH) 353 nm (ε= 17,800), 276 (ε= 9,400), 223 (ε= 19,000); IR ν_{max} ($CDCl_3$) 3466, 1643, 1616, 1588 cm^{-1}; HRMS:

m/z (%) 185.0838 (M^+, 26, $C_{12}H_{11}NO$ requires 185.0841), 170 (40), 144 (100), 115 (15), 89 (12); ^1H NMR (d_6-acetone) δ8.6 (1H, br s), 7.95 (1H, d, J= 7 Hz), 7.85 (1H, d, J = 16 Hz), 7.88 (1H, d, J= 3 Hz), 7.5 (1H, d, J= 7 Hz), 7.2 (2H, m), 6.75 (1H, d, J = 16 Hz), 2.3 (3H, s); ^{13}C NMR ($CDCl_3$) δ198.7, 137.3, 137.2, 129.2, 125.3, 123.5, 123.4, 121.7, 120.5, 113.7, 111.8, 27.4.

5-(indol-3-yl)pent-3(E)-ene-2-one, 21

Yield: 2.8 mg from 7.8 g $CHCl_3$ solubles; yellow oil; UV λ_{max} (EtOH) 290 nm (ε= 5,200), 282 (ε= 5,900), 224 (ε= 20,700); IR ν_{max} ($CDCl_3$) 3481, 2282, 1661 cm^{-1}; HRMS: m/z (%) 199.1005 (M^+, 79, $C_{13}H_{13}NO$ requires 199.0997) 184 (19), 156 (100), 130 (46), 117 (13), 89 (7); ^1H NMR (d_6-acetone) δ10.1 (1H, br s), 7.5 (1H, d, J= 8 Hz), 7.3 (1H, d, J= 8 Hz), 7.19 (1H, s, J= 2.1 Hz), 7.10 (1H, t, J= 8, 7 Hz), 7.0 (2H, m, J = 6, 8, 16 Hz), 6.1 (1H, d, J = 16 Hz), 3.7 (2H, dd, J = 6, 23 Hz), 2.16 (3H, s); ^{13}C NMR (d_6-acetone) δ198.1, 147.5, 137.8, 132.1, 128.3, 124.5, 123.8, 122.3, 119.6, 119.3, 112.2, 112.1, 29.0, 26.8.

3-(hydroxyacetyl)indole, 23

Yield: 7.8 mg from 7.8 g $CHCl_3$ solubles; white crystalline solid; UV λ_{max} (EtOH) 295 nm (ε= 3,500), 260

(ε= 2,800), 239 (ε= 3,700), 220 (ε= 4,000); HRMS: m/z (%) 175.0651 (M^+, 3, $C_{10}H_9NO_2$ requires 175.0663), 144 (100), 130 (21), 116 (20), 89 (19); ^1H NMR (CD_3OD) δ8.2 (1H, dd), 8.1 (1H,s), 7.4 (1H, dd), 7.15-7.25 (2H, m), 4.69 (2H, s); ^{13}C NMR (CD_3OD) δ196.0, 138.2, 134.0, 126.9, 124.4, 123.3, 122.6, 114.8, 112.9, 66.3.

1-methyl carbazole, 24

Yield: 2.3 mg from 5.6 g CCl_4 solubles; yellow solid; UV λ_{max} (EtOH) 336 nm (ε= 1,600), 325 (ε= 1,800), 292 (ε= 7,100), 257 (ε= 9,200), 236 (ε= 18,800); HRMS: m/z (%) 181.0871 (M^+, 100, $C_{13}H_{11}N$ requires 181.0892); ^1H NMR (d_6-acetone) δ8.08 (1H, d), 7.93 (1H, d), 7.50 (1H, d), 7.35 (1H, t), 7.19 (1H, d), 7.16 (1H, t), 7.08 (1H, t), 2.56 (3H, s); ^{13}C NMR ($CDCl_3$) δ139.4, 138.9, 126.4, 125.6, 123.8, 120.9, 119.7, 119.6, 119.5, 117.9, 110.7, 16.8.

1-acetyl-β-carboline, 25

Yield: 4.5 mg from 13.41 g CCl_4 and $CHCl_3$ solubles; yellow crystalline solid; UV λ_{max} (EtOH) 378 nm (ε= 1,500), 306, 283 (ε= 7,700), 252, 224 (ε= 17,500); IR ν_{max} ($CDCl_3$) 3444, 1671 cm^{-1}; HRMS: m/z (%) 210.0756 (M^+, 89, $C_{13}H_{10}N_2O$ requires 210.0793), 182 (37), 168 (100), 140 (33); ^1H NMR (d_6-acetone) δ8.5 (1H, d, J = 5 Hz), 8.4 (1H, d, J = 5

Hz), 8.3 (1H, d, J = 8 Hz), 7.8 (1H, d, J = 8 Hz), 7.6 (1H, t, J = 7.3, 8 Hz), 7.3 (1H, t, J = 7.3, 8 Hz), 2.8 (3H, s); ^{13}C NMR ($CDCl_3$) δ203.3, 141.1, 138.2, 136.1, 135.4, 131.5, 129.3, 121.8, 120.7, 120.6, 119.1, 112.0, 25.9.

2-phenylacetamide, 26

Yield: 5.8 mg from 7.8 g $CHCl_3$ solubles; white crystalline solid; UV λ_{max} (EtOH) 265 nm (ε= 200), 258 (ε= 300), 253 (ε= 300), 222 (ε= 1,200); IR ν_{max} ($CDCl_3$) 3519, 3412, 3036, 1687, 1586 cm^{-1}; HRMS: m/z (%) 135.0706 (M^+, 19.0, C_8H_9NO requires 135.0684), 92 (100); ^1H NMR (d_6-acetone) δ7.3-7.4 (5H, m), 5.85 (1H, br s), 5.50 (1H, br s), 3.6 (2H, s); ^{13}C NMR ($CDCl_3$) δ173.5, 134.8, 129.4 (2 carbons), 129.0 (2 carbons), 127.4, 43.3.

2-phenylacetic acid, 27

Yield: 14.9 mg from 5.6g CCl_4 solubles; white crystalline solid; UV λ_{max} (EtOH) 326 nm (ε= 1,000), 265 (ε= 300), 258 (ε= 400), 223 (ε= 1,800); IR ν_{max} ($CDCl_3$) 3010 (broad), 1710 cm^{-1}; HRMS: m/z (%) 136.0531 (M+, 42, $C_8H_8O_2$ requires 136.0524), 91 (100); ^1H NMR ($CDCl_3$) δ7.2-7.4 (5H, m), 3.65 (2H, s); ^{13}C NMR ($CDCl_3$) δ177.4, 133.3, 129.3 (2 carbons), 128.6 (2 carbons), 127.3, 41.0.

4-(2′,3′,4′-trimethylphenyl)but-3(E)-en-2-one, 28

Yield: 2.0 mg from 5.6 g CCl_4 solubles; yellow oil; IR ν_{max} (CCl_4) 1673 cm^{-1}; HRMS: m/z (%) 188 (M^+, 8), 173.0975 (100, $C_{12}H_{13}O$, M^+-15, requires 173.0966), 129 (15); ^1H NMR (d_6-acetone) δ7.9 (1H, d, J = 16 Hz), 7.37 (1H, d, J= 8 Hz), 7.04 (1H, d, J= 8 Hz), 6.57 (1H, d, J = 16 Hz), 2.34 (3H, s), 2.32 (3H, s), 2.28 (3H, s), 2.20 (3H, s); ^{13}C NMR ($CDCl_3$) δ198.4, 142.4, 139.0, 136.2, 135.9, 131.6, 127.9, 127.8, 123.9, 27.8, 21.2, 16.0, 15.9.

2-phenylethenyl acetate, 29

Yield: 2.6 mg from 7.8 g $CHCl_3$ solubles; yellow oil; UV λ_{max} (EtOH) 285 nm (ε= 9,900), 242 (ε= 4,200), 224 (ε= 7,500); IR ν_{max} ($CHCl_3$) 1760, 1670, 1605 cm^{-1}; HRMS: m/z (%) 162.0658 (M+, 36, $C_{10}H_{10}O_2$ requires 162.0681), 147 (14), 133 (100), 103 (42), 91 (37), 77 (52); ^1H NMR (d_6-acetone) δ7.69-7.66 (m), 7.60 (1H, d, J= 16.3 Hz), 7.44-7.42 (m), 6.77 (1H, d, J= 16.3 Hz), 2.33 (3H, s); ^{13}C NMR ($CDCl_3$) δ143.4, 134.4, 130.7, 130.5, 129.0, 128.2, 127.2, 123.0, 27.5.

2-(2′,4′-dibromophenoxy)-3,5-dibromophenol, 47

Yield: 2.8 mg from 5.6 g CCl_4 solubles; UV λ_{max} (EtOH) 291 nm (ε= 4,900), 284 (ε= 4,700), 224 (ε= 25,200);

HRMS: M+ 497.7113 ($C_{12}H_6{}^{79}Br_4O_2$ requires 497.7101); ^{1}H NMR (d_6-acetone) δ7.80 (1H, d, J= 2.4 Hz), 7.41 (1H, dd, J= 8.8, 2.4 Hz), 7.28 (1H, d, J= 2.2 Hz), 7.19 (1H, d, J= 2.2 Hz), 6.56 (1H, d, J= 8.8 Hz); ^{13}C NMR ($CDCl_3$) δ152.1, 150.4, 138.7, 136.2, 131.5, 127.7, 119.8, 119.7, 117.2, 116.0, 115.9, 112.7.

1-hydroxy-2,3,6,7-tetrabromodibenzo-p-dioxin, 49

Yield: 0.9 mg from 5.6 g CCl_4 solubles; UV λ_{max} (EtOH) 292 nm (ε= 4,300), 239 (ε= 29,300), 224 (ε= 29,600); HRMS: M+ 511.6884 ($C_{12}H_4{}^{79}Br_4O_3$ requires 511.6874); ^{1}H NMR (d_6-acetone) δ7.43 (1H, d, J= 1.9 Hz), 7.12 (1H, d, J= 1.9 Hz), 7.05 (1H, s).

1-hydroxy-2,6,8-tribromodibenzo-p-dioxin, 53

Yield: 0.6 mg from 5.6 g CCl_4 solubles; UV λ_{max} (EtOH) 289 nm (ε= 7,300), 230 (ε= 38,700), 220 (ε= 41,700); HRMS: M+ 433.7797 ($C_{12}H_5{}^{79}Br_3O_3$ requires 433.7789); ^{1}H NMR (d_6-acetone) δ7.43 (1H, d, J= 2.4 Hz), 7.12 (1H, d, J= 2.4 Hz), 7.07 (1H, d, J= 8.9 Hz), 6.64 (1H, d, J= 8.9 Hz).

1-hydroxy-3,7,9-tribromodibenzo-p-dioxin, 55

Yield: 0.6 mg from 5.6 g CCl_4 solubles; UV λ_{max}

(EtOH) 233 nm (ε= 32,800), 222 (ε= 38,400); HRMS: M+ 433.7797 ($C_{12}H_5{}^{79}Br_3O_3$ requires 433.7789); ^{1}H NMR (d_6-acetone) δ7.42 (1H, d, J= 2.3 Hz), 7.09 (1H, d, J= 2.1 Hz), 6.83 (1H, d, J= 2.3 Hz), 6.72 (1H, d, J= 2.1 Hz).

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